MONOGENIC HYPERCHOLESTEROLEMIA IN SOUTH AFRICANS: FAMILIAL HYPERCHOLESTEROLEMIA IN INDIANS AND FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

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CERTIFICATE OF SUPERVISOR

In terms of Paragraph GP8 of "General rules for the degree of PhD", I, as supervisor of the candidate, David Chaim Rubinsztein, certify that I approve of the incorporation in this thesis of material that is in press for publication.
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SUMMARY

LDL-receptor mutations and familial defective apolipoprotein B-100 (codon 3500) (FDB), the known causes of monogenic hypercholesterolemia (MH), have similar clinical features. The nature of the mutations responsible for MH in South Africans of Indian origin was previously unknown. Similarly, the mutations in the LDL-receptor gene of a South African Black FH homozygote had also not been characterised. The aim of this thesis was to identify and analyse the LDL-receptor mutations in the Indian homozygotes NS, D, AV and AA and in the Black homozygote JL. In addition, the possible importance of FDB as a cause of MH in South Africans was also assessed.

The patient NS was characterized as having two "Null" LDL-receptor alleles. His skin fibroblasts expressed no detectable LDL-receptor protein and very low levels of LDL-receptor mRNA of approximately normal size. Since NS's LDL-receptor promoter sequence was normal, his alleles are likely to harbour exonic point mutations or minor rearrangements that cause premature stop codons.

The patient D was found to be a heteroallelic homozygote. Two new point mutations in the LDL receptor, Asp_{69}-Tyr and Glu_{119}-Lys, were identified. D's fibroblasts expressed about 30% of the normal surface complement of receptors that bound LDL poorly. This low number could at least be partially explained by their decreased stability. These mutations were not

identified in any other Indian FH or hypercholesterolemic patients.

Patients AV and AA were both shown to be homoallelic homozygotes for the Pro_{664}-Leu mutation. This mutation was identified in 4 unrelated Muslim families of Gujerati origin suggesting that the mutation arose from this area in India. Contrary to previous reports (Knight et al. 1990, Soutar et al. 1989), neither LDL nor ß-VLDL binding were shown to be affected by this mutation. These mutant receptors were rapidly degraded. Thus the disease FH in these subjects is presumably due to the low steady-state level of mature receptors that are functionally normal but exhibit accelerated turnover.

The Pedi FH homozygote, JL, expressed very few LDL receptors due to decreased receptor synthesis associated with low mRNA levels and not due to enhanced degradation. One of JL's LDL receptor alleles has a 3 b.p. deletion in repeat 1 of the promoter (G. Zuilani, H. Hobbs and L.F. de Waal, personal communication). The nature of the defect in his other allele is unknown.

The importance of FDB as a cause of monogenic hypercholesterolemia in the South African Indian, "Coloured" and Afrikaner populations was determined by screening hypercholesterolemic subjects with or without xanthomata. The absence of FDB in such patients, in whom the relevant common

or founder South African mutations were excluded, suggested that this disorder was rarer in these groups than in North America and Europe.

FDB was identified in two different families of mixed British and Afrikaner ancestry. One family contained individuals who were heterozygous for the FDB mutation, as well as the FH Afrikaner-1 and the FH Afrikaner-2 LDL-receptor mutations. In addition, 4 compound heterozygotes, who had both FDB and the FH Afrikaner-1 mutation and one individual who inherited all 3 defects, were identified. This family allowed us to characterise the compound heterozygotes with one mutant LDL-receptor allele and FDB as having a condition that was probably intermediate in severity between the FH heterozygote and homozygote states.

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ABBREVIATIONS

amps = ammonium persulphate

Apo = Apolipoprotein

ß-VLDL = Beta migrating very low density lipoprotein

BSA = Bovine serum albumin

CAT = Chloramphenicol acetyltransferase

CHO = Chinese hamster ovary

DEPC = Diethylpyrocarbonate

DiI = 1, 1' dioctadecyl-3,3,3',3' tetramethylindocyanine perchlorate

DMEM = Dulbecco's modification of Eagle's Minimum Essential Medium

ER = Endoplasmic Reticulum

FCS = Fetal calf serum

FDB = Familial defective apolipoprotein B-100.

FH = Familial hypercholesterolemia

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HMG-CoA = 3-hydroxymethyl-3-methylglutaryl coenzyme A

HSV = Herpes simplex virus

IDL = Intermediate density lipoprotein

LB = Luria/Lennox broth

LDL = Low density lipoprotein

LPDS = Lipoprotein deficient serum

MEM = Eagle's Minimal Essential Medium buffered with Earle's salts

PBS = Phosphate-buffered saline

PCR = Polymerase chain reaction

SSCP = Single-strand conformational polymorphism

TEMED = N,N,N',N'-tetramethylethylenediamine

TK = Thymidine kinase

TLC = Thin layer chromatography

VLDL = Very low density lipoprotein

X-Gal = 5-Bromo-4-chloro-3-indolyl-ß-D-galactoside

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CHAPTER 1

MONOGENIC HYPERCHOLESTEROLEMIA: A REVIEW OF NORMAL AND MUTANT LDL-RECEPTOR BIOLOGY AND FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

1.1 INTRODUCTION TO LIPOPROTEIN METABOLISM AND MONOGENIC HYPERCHOLESTEROLEMIA

1.1.1 NORMAL LIPOPROTEIN METABOLISM

Cholesterol plays important roles in vertebrate structure and metabolism as a constituent of cell membranes and as a precursor for steroid hormones and bile acids (Myant 1990). Two different pathways transport cholesterol in the blood. The "exogenous" pathway delivers dietary cholesterol mainly to the liver. Chylomicron particles containing triglycerides and cholesterol absorbed from the intestine are secreted into the lymphatic system and enter the circulation via the thoracic duct. The triglycerides in these large lipoproteins are hydrolysed by lipoprotein lipase on the vascular endothelium. The resultant smaller chylomicron remnants are rapidly removed from the blood mainly by receptors on parenchymal liver cells (Shepherd and Krauss 1991). These receptors are probably distinct from the LDL receptor since chylomicron remnant clearance is unimpaired in FH homozygote subjects with severe LDL-receptor deficiency (Rubinsztein et al. 1990). The LDL-receptor related protein (LRP), the α_2 macroglobulin receptor, might be the chylomicron remnant receptor (Brown et al. 1991).

The "endogenous" cholesterol pathway is concerned with the transport in the circulation of apo B-100 containing lipoproteins secreted by the liver. These particles are initially known as very low density lipoproteins (VLDL). The nascent VLDL particles contain apo B-100, apo E and a small amount of apo C. As soon as the VLDL enters the circulation it acquires additional apo E and apo C derived from HDL metabolism. The VLDL triglycerides are lipolysed by lipoprotein lipase to yield smaller particles called intermediate density lipoproteins (IDL). These IDL can either be further lipolysed by lipoprotein lipase or hepatic lipase to form low density lipoproteins (LDL) or can be directly removed from the circulation via the interaction of apo E with LDL receptors. LDL particles, which carry most of the plasma cholesterol, are removed from the circulation mainly by hepatic LDL receptors interacting with their sole apolipoprotein, apo B-100. Although approximately 70% of LDL catabolism is via the liver, the major route of cholesterol exit from the body, significant LDL clearance from the blood is mediated by the small intestine, skeletal muscle, kidney and spleen (Myant 1990). A small proportion of LDL in normal individuals is cleared by non-LDL-receptor pathways. Although most of the cholesterol eliminated from the body is via the hepatic route, some cholesterol is lost in sebum and in desquamated intestinal and skin cells.

1.1.2 MENDELIAN DISEASES THAT RESULT IN IMPAIRED LDL METABOLISM

Two related syndromes have been characterised where defective removal of LDL via LDL receptors is the primary defect. Familial Hypercholesterolemia (FH) results from mutations at

the LDL-receptor gene locus that affect either the number and/or function of the LDL-receptor proteins. This causes two different perturbations in the endogenous cholesterol pathway that result in hypercholesterolemia. Firstly, an overproduction of LDL from IDL results from impaired direct IDL removal from the circulation and secondly the rate of LDL removal is decreased (James et al. 1989). The clinical severity of this disease is milder in patients with one mutant gene (heterozygotes) than in those with two (homozygotes). Heterozygotes have approximately 50% of the normal number of functional LDL receptors on their cells (a simplification). Their plasma cholesterol levels are about twice as high as normal and many have evidence of premature coronary artery disease: about 50% of FH heterozygote males have coronary heart disease by age 50. The situation in homozygotes who have two mutant alleles is far more severe: Plasma cholesterol concentrations are raised up to 5x the normal levels and coronary artery disease often manifests in childhood with death from a heart attack occuring frequently before the age of 25 (Thompson et al. 1989).

An alternative gene defect that causes decreased LDL clearance is the glutamine for arginine substitution at codon 3500 of apo B. Apo B-100 is the sole apoprotein on LDL and is the ligand on the particle that binds to the LDL receptor. The LDL that carry apo B with the arg-gln mutation at codon 3500 bind very poorly to LDL receptors and are cleared abnormally slowly from the circulation. This condition,

called familial defective apo B-100 (FDB), is inherited in an autosomal dominant fashion (Innerarity et al. 1990). FDB in its heterozygous state is clinically very similar to heterozygous FH (Rauh et al. 1992).

1.2 THE INTINERARY OF THE LDL RECEPTOR

LDL-receptor proteins are synthesized on polyribosomes and are co-translationally inserted into the endoplasmic reticulum where high-mannose N-linked glycosylation occurs. The receptor is then transported to the smooth endoplasmic reticulum where O-linked N-acetyl-galactosamine residues are thought to be added to the receptor (Myant 1990). At this point the precursor receptor has an apparent MW of 120 kDa. After transport to the Golgi, where the high-mannose N-linked sugars are trimmed and converted to complex chains and galactose and sialic acid residues are added to the O-linked chains, the receptor attains its mature form, MW 160 kDa. This process takes 15-30 minutes. It seems that the increase of the apparent MW from 120 kDa precursor to the 160 kDa mature receptor is largely due to the addition of galactose and sialic acid residues to O-linked chains in the trans Golgi (Tolleshaug et al. 1982, Cummings et al. 1983).

Cummings et al. (1983), in their extensive analysis of the glycosylation of the LDL-receptor, demonstrated considerable heterogeneity of O-linked glycosylation. It is likely that the O-linked glycosylation is heterogeneous within each receptor but the possibility exists that O-linked

glycosylation varies between receptors i.e. receptors have different O-linked chains at homologous sites. These authors found that each receptor had only one N-linked sugar chain. However, there were more than 2 different types of N-linked sugar chains in the mature LDL receptors from each of the cell types studied. This suggested that there are differently glycosylated populations of LDL receptors produced by normal cells. The possibility that functional heterogeneity of normal LDL-receptor sub-populations might exist has not been investigated. Some mutant receptors, e.g. FH Afrikaner-1 (Fourie et al. 1988), however, do manifest functionally heterogeneous sub-populations of receptors that might be subtly differently glycosylated.

The conversion of precursor to mature receptors is quantitatively virtually complete in normal cells (Tolleshaug et al. 1982, Knight et al. 1989). This implies that hardly any precursor degradation occurs. It is possible that a "pre-precursor" form of the LDL receptor exists that is not immunoprecipitable by anti-LDL-receptor antibodies or is not immunoprecipitated at 120 kDa. Alternatively, the steady-state pool of these pre-precursor receptors could be very small. These pre-precursor receptors would possibly include proteins that are folding and re-folding in order to attain their correct conformation(s). Nobody knows what the kinetics of "pre-precursor" to precursor conversion are or whether this conversion is completed for all molecules. It is possible that the extent of "pre-precursor" to precursor

conversion is impaired by some of the many mutations that seem to affect normal protein folding.

From the Golgi the receptor is transported to the cell surface, where it can bind lipoproteins that bear its ligands apo B-100 and/or apo E. The receptors in fibroblasts are particularly concentrated in specific regions of the plasma membrane called coated pits that mediate their endocytosis (Anderson et al. 1977).

LDL receptors are internalized into the cell whether or not they have bound ligand (Anderson et al. 1982, Basu et al. 1981). As endocytosis proceeds, the pH in the vesicles is lowered by a proton-driven pump (Tycko and Maxfield 1982). In the compartment of uncoupling of the receptor and ligand (CURL), the pH is sufficiently low for the receptor and ligand to dissociate. At this point they part ways. The freed receptor can return to the cell surface where it can undergo many further rounds of endocytosis (Brown et al. 1983). Each round trip takes about 12 minutes (Basu et al. 1981). The ligand is delivered to lysosomes where its apoproteins are degraded and the cholesterol esters are hydrolysed. The liberated free cholesterol is transported out of the lysosome and will have many important regulatory consequences in the cells. It inhibits the synthesis of LDL receptors (Brown and Goldstein 1975). It stimulates ACAT (the enzyme that forms intracellular cholesterol esters) (Goldstein et al. 1974) and inhibits the activity of HMG-CoA

reductase, the rate-limiting enzyme in the cell's de novo cholesterol synthesis pathway (Brown et al. 1974).

1.3 THE GENETIC ORGANISATION AND PROTEIN STRUCTURE OF THE LDL RECEPTOR

The 860 amino acid LDL receptor (Yamamoto et al. 1984) is a multi-domain protein, with components of diverse evolutionary origin (Südhof et al. 1985). The LDL receptor is an ancient protein that is highly conserved across many species ranging from Xenopus to humans (Mehta et al. 1991). The 45 kB gene (19p 13.1 - p 13.3) (Lindgren et al. 1985), containing 18 exons and 17 introns, is transcribed into a 5.3 kB mRNA (Südhof et al. 1985). The exons are arranged in a modular fashion where each exon or group of exons defines a distinct domain of the receptor (see Fig. 1.1).

Exon 1 codes for a short 5' untranslated region and a 21 amino acid signal sequence that aids in the co-translational translocation of the protein into the ER. This region is cleaved off during the translocation process to yield a mature protein of 839 amino acids.

Exons 2-6 code for the LDL-receptor's ligand-binding domain. This domain consists of 7 40-amino acid repeats homologous to the complement factor 9. Each repeat has 6 cysteines that are thought to be involved in intra-repeat disulphide bonds.

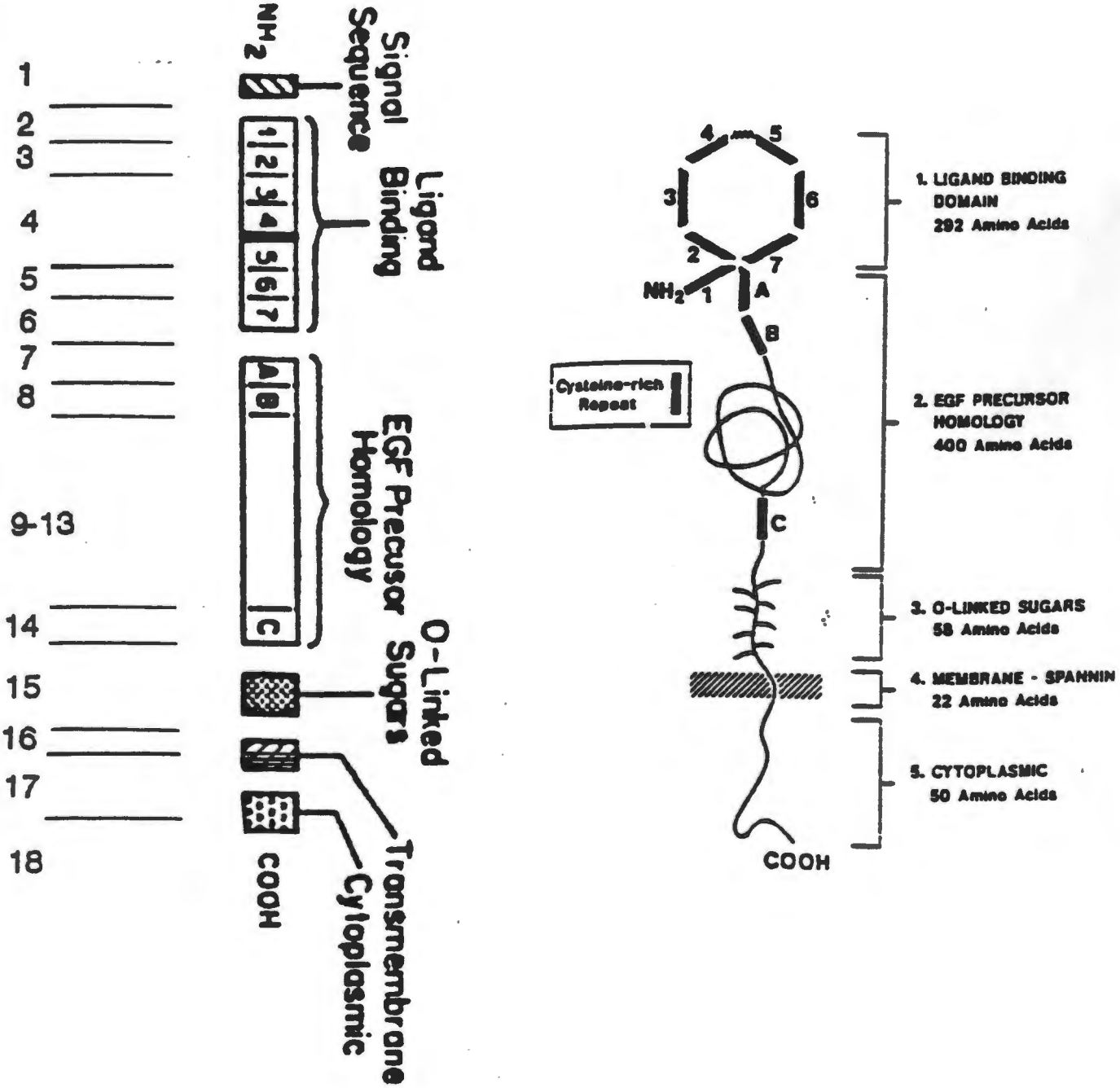


Fig. 1.1: Schematic diagram of the LDL-receptor protein and gene showing relationship of exons to specific domains (from Esser et al. 1988 and Russell et al. 1989) with permission of the authors and the American Society for Biochemistry and Molecular Biology.

Exons 7-14 code for the EGF-precursor homology domain, so named because of its DNA sequence similarity to the mouse EGF-precursor molecule. Exons 7, 8 and 14 code for 40-amino acid repeats of distinct evolutionary origin from those found in the ligand-binding domain. These repeats (named A,B and C) also contain 6 disulphide-bonded cysteines (Südhof et al. 1985). Repeats A and B are separated from repeat C by 280 amino acids in which 5 repeats of the motif YWTD are found. This EGF-precursor homology domain is thought to have 4 functions: Repeat A is necessary for optimal LDL (Apo B) binding to the receptor (Esser et al. 1988). The domain as a whole mediates acid-dependent dissociation of the receptor from its ligand in endosomes (Davis et al. 1988) and the YWTD repeats are thought to play a role in protecting the receptor from rapid degradation (Hobbs et al. 1990). The intactness of the domain is crucial for normal folding of the receptor and transport from the ER to the Golgi (Hobbs et al. 1990).

Exon 15 codes for a region of 48 amino acids including 18 serines and threonines that are potential attachment sites for O-linked sugars (Südhof et al. 1985). The deletion of this region in Chinese hamster ovary cells has no deleterious consequences on receptor function (Davis et al. 1986). However, there is a natural mutation, FH Tonami-1, where this domain is deleted, that manifested slow processing of the precursor to the mature form (Mabuchi et al. 1990).

Exons 16 and the start of exon 17 code for the membrane spanning domain, and the 3' end of exon 17 and the 5' end of exon 18 code for the cytoplasmic tail of the receptor (Südhof et al. 1985). The cytoplasmic domain contains amino acids that are important determinants for the clustering of receptors in coated pits. Mutations to crucial amino acids in this region result in receptors that bind ligand but do not get internalized to any significant extent (Davis et al. 1987, Chen et al. 1990). In addition to amino acid signals that ensure normal clustering in coated pits, the LDL-receptor tail has distinct sequences that direct the receptors to the apical and/or basolateral domains of polarised cells (Pathak et al. 1990).

LDL-receptor expression is largely regulated by the level of intracellular unesterified cholesterol. There are, however, other hormonal influences that regulate LDL-receptor levels that I will not review here (See Soutar and Knight 1990).

The transcription of the LDL-receptor gene is modulated by proteins that bind to DNA sequences within 200 base pairs upstream of the translation initiation codon. This promoter contains a TATA like sequence and 3 16-base pair imperfect direct repeats (Südhof et al. 1987) that seem to be necessary for maximal transcription. Repeats 1 and 3 bind the transcription factor Sp-1 (Dawson et al. 1988). The 2nd repeat is the sterol regulatory element that is a positive transcriptional element in the absence of sterols (Südhof et

al. 1987), but is also the site of inhibition of transcription in the presence of sterols (Dawson et al. 1988). The regulation of steady-state levels of receptors was thought to occur almost entirely at the level of transcription. Recent work has suggested that sterol regulation can take place at a post-transcriptional level as well (Tam et al. 1991, Sharky et al. 1990). The details of this mechanism have not been further elucidated.

The degradation pathways of the LDL receptor have not been fully characterised. The mature fibroblast receptor has a half-life of 10-12 hours that is unaffected by the presence or absence of sterols (Casciola et al. 1988). This suggests that sterol regulation of receptor levels is occurring almost exclusively at the level of receptor synthesis (transcription and maybe translation). Since the degradation of the mature receptor was not affected by the lysosomotropic agent leupeptin, the initial steps in this process are unlikely to be lysosomal (Casciola et al. 1989). The degradation process depends on the presence of short-lived proteins since the half-life of receptors is increased in cells treated with cycloheximide (Casciola et al. 1988).

1.4 VARIATION IN "NORMAL" LDL-RECEPTOR GENES

Restriction fragment length polymorphism (RFLP) analysis of LDL-receptor genes has yielded 22 different polymorphic sites (Hobbs et al. 1990) and 123 different haplotypes (Leitersdorf et al. 1989a). Because there is linkage disequilibrium

between many of the sites, the analysis of 5 selected sites is probably sufficient as the cumulative heterozygosity index of these sites is 84% and the addition of a further 5 sites only increases the heterozygosity index to 86% (Hobbs et al. 1990). There have been some attempts to link certain RFLPs to variation in cholesterol levels in normolipidemic populations. In 3 different populations the presence of a Pvu II cutting site was correlated with reduced cholesterol levels (Pedersen and Berg 1988, Schuster et al. 1990, Humphries et al. 1991). This suggests that some factor in linkage disequilibrium with the Pvu II RFLP site in the LDL-receptor gene, that possibly alters LDL-receptor structure or expression, results in lower cholesterol levels.

1.5 LDL-RECEPTOR MUTATIONS

More than 40 different mutant LDL-receptor genes have been detected to date (Hobbs et al. 1990). These have been classified into 5 classes by the Dallas group depending on their phenotypic defect (Hobbs et al. 1990). Class 1 mutations produce no immunoprecipitable receptors. The null alleles that produce no mRNA are often large deletions that remove the gene's promoter. Other null alleles produce mRNA that is reduced in quantity. This can result from nonsense mutations or deletions that create premature stop codons. The mRNA levels are thought to be low in these cases due to the lack of ribosomal binding to a large stretch of mRNA exposing it to increased degradation (Trecartin et al. 1981). Null mutations could possibly also result from very rapid

degradation of the LDL-receptor precursor (Hobbs et al. 1990).

Class 2 mutations produce proteins that are slowly transported from the ER to the Golgi. This is manifested in a delay in conversion of the precursor receptor (120 kD) to its mature form (160 kD). These mutations have been classified into either complete (type 2A) or partial (type 2B) blocks of transport. The type 2A mutations described thus far occur in the EGF-precursor homology domain (Hobbs et al. 1990) or result from the deletion of the O-linked sugar domain (Mabuchi et al. 1990). Various point mutations and small deletions in the ligand-binding domain result in type 2B mutations (Hobbs 1990). The distinction between type 2A and 2B mutations is maybe not such a simple matter. The absence of any mature receptors in these 2A mutations might be due to a complete block in transport of precursor receptors to the Golgi. Alternatively, since it is known that many mutations in the EGF-precursor domain also cause rapid degradation of the mature receptor (eg FH-Afrikaner-2, Fourie et al. 1988, and FH-Cape Town-2, van der Westhuyzen et al. 1991), it is possible that the absence of detectable mature receptors is a result of very rapid degradation of the mature receptors and not due to an absolute block in transport. The slow transport phenomenon is fairly widespread among human genetic diseases and is probably the result of improper folding of mutant proteins in the ER. These malfolded molecules get subsequently retained in the ER

by proteins such as Bip (Gething and Sambrook 1992). Partially impaired transport from the ER to the Golgi is not sufficient to cause a lower than normal number of cell surface LDL receptors. A low cell-surface receptor complement will presumably only manifest if, in addition to slow transport, there is either decreased receptor protein synthesis or enhanced absolute degradation of either the precursor or mature forms of the receptor. If there is a low fractional degradation rate of normal precursor receptors that is not usually noticeable, it might be a mechanism that becomes increasingly prominent in determining mature receptor pool sizes in slowly transported mutations. In these cases, the absolute precursor degradation rate can be greatly increased as the precursor receptor pool expands due to impaired transport from the ER to the Golgi, even if the fractional degradation rate is unaltered. This scenario is probably not operative in all type 2B mutations, since the conversion of precursors to mature receptors was quantitatively virtually complete in the slowly processed FH Zambia mutation (Knight et al. 1989).

Type 3 mutations exhibit impaired ligand binding. Mutations in repeats 2 to 7 of the ligand-binding domain and repeat A of the EGF-precursor homology domain affect LDL (apo B) binding, while only mutations in repeat 5 in the ligand-binding domain seem to impair ß-VLDL (Apo E) binding (Esser et al. 1988, Russell et al. 1989). These mutations will be

considered in greater detail in a later section: Ligand-binding requirements of the LDL receptor.

Mutant receptors that cannot internalise bound ligand normally are grouped in Class 4. These mutations are either deletions of the transmembrane domain and the cytoplasmic tail that result in secretion of the LDL receptor from the cell (reviewed by Hobbs et al. 1990) or, more commonly, mutations in the cytoplasmic tail that affect the clustering of receptors in coated pits and subsequent internalisation (Davis et al. 1987) The sequence NPVY (Asn-Pro-Val-Tyr), starting at amino acid 804, is found in LDL receptors from 6 species. The NP and Y from this tetrameric sequence and the phenylalanine at 802 are the important signals that ensure normal receptor clustering in coated pits. A single amino acid substitution or deletion at one of these crucial sites can result in an internalisation-defective phenotype (Chen et al. 1990). This process is mediated by the interaction of the receptor tail with adaptor molecules that are in turn associated with the clathrin coat of coated pits (Pearse 1988).

Recent findings suggest a correlation between effective internalisation and the presence of a reverse-turn conformation in the LDL-receptor cytoplasmic tail (Bansai 1991). It is likely that the aromatic amino acid Tyr (Y) in the NPVY motif plays a crucial role in maintaining the reverse-turn conformation (Bansai and Gierasch 1991) and that

this same Tyr is also recognised by adaptor molecules in the context of its tight turn (Eberle et al. 1991).

In 1990 a fifth class of mutations was added to the above four. These were defined as recycling-deficient receptors that did not separate from their bound ligands after being internalised in endosomes. These receptors are trapped in the cell and are unable to return to the cell surface. The degradation rate of these (mature) receptors is often increased as a consequence of this phenomenon. Certain mutations in the EGF-precursor domain manifest this phenotype (Hobbs et al. 1990). Perhaps the entrance criteria for this class of mutation should be extended to include mutations that result in increased degradation of mature and/or precursor receptors in the absence of ligand. The defect that results in increased turnover of these mutant receptors is not primarily impaired acid-dependent ligand-receptor dissociation in endosomes. Mutations that result in unstable receptors are probably quite common, since, in the absence of a synthetic defect, increased degradation would be the only explanation for a decreased surface receptor complement in class 2B receptor mutants (assuming they have not been secreted from the cell). If one assumes that the transport of receptors (mutant and normal) from the ER to the Golgi is a first order process, then even in a severe type 2B mutuation there would be a normal absolute number of receptors transported from the ER to the Golgi per unit time, as long as no precursor degradation occurs. At steady-state

the number of receptor molecules entering the ER would equal the number of those leaving. The ER pool of receptors in a type 2B defect would be greatly increased until the concentration of receptors would be high enough to allow a normal number of receptors to be transported per unit time to the Golgi in spite of the reduced fractional transport rate.

1.6 LIGAND-BINDING REQUIREMENTS OF THE LDL RECEPTOR

Apolipoproteins B-100 and E mediate the binding of lipoproteins to the LDL receptor (Goldstein et al. 1985, Mahley and Innerarity 1983). Apo B-100 is responsible for LDL binding while apo E containing lipoproteins bind via that apoprotein whether apo B-100 is present or not (Innerarity 1990). Canine HDL_C or rabbit ß-VLDL, which are both induced by cholesterol feeding, are the ligands that are traditionally used to measure the LDL receptor's ability to bind apo E (Innerarity 1990).

The interaction of the LDL receptor and its ligands is thought to be ionic and Ca^{2+} dependent. At the carboxy-terminus of each of the ligand-binding repeats is a highly conserved triad of negatively charged amino acids (Ser-Asp-Glu) that are thought to play a major role in binding by interacting with positively charged areas of Apo B and Apo E (Goldstein et al. 1985). The classic understanding is that the stochiometry of LDL to receptor binding is 1:1 while 3-4 receptors bind to one HDL_C particle: This is probably due to the fact that each LDL particle contains one apo B-100

molecule, while there are multiple copies of apo E on each HDL_C molecule. Because each HDL_C particle binds to many LDL receptors, the affinity of this lipoprotein for the LDL receptor is much higher than that of LDL. Studies have suggested that the LDL receptor might in fact exist and act as a multimer (Reviewed by Innerarity 1990).

The structural requirements of the LDL receptor that enable lipoproteins to bind have been determined largely through the observations of the behaviour of natural and artificial mutant receptors. The reasoning used in this approach has been that if a particular mutation impairs the normal function of the receptors, then the area that has been altered is important for that function. The converse reasoning has also been used: if the mutation has no effect, then the area that has been mutated is not crucial for the particular function one is determining. Although this approach is traditionally used, I believe one should be very circumspect when interpreting its results. The difference or similarity in behaviour between a mutated protein (let's say for simplicity's sake with a point mutation) and the wild-type protein can be subjected to many interpretations. The point mutation has removed an amino acid that might have had a specific function. How does one define the function of an amino acid in the LDL receptor? Since this protein has not been crystallised and is therefore not amenable to structural investigations using X-ray crystallography, one has to look at functional characteristics. If the mutation causes a

functional aberration by virtue of the amino acid being removed, is this because the amino acid being removed has a specific role (eg. a glycosylation site) or is it because the 2° and 3° protein structure has been distorted. This issue is particularly problematical in deletion mutations where entire domains of the receptor are removed and the 2° and 3° structure of the protein are certainly affected. In addition, the ability of the LDL receptor to form multimers might be affected.

The other major difficulty in the interpretation of mutagenesis experiments is whether the observed effect of the mutation is the result of the removal of the wild-type amino acid (in the case of a point mutation) or is it the consequence of the mutant amino acid that has been introduced. This problem can be addressed but not always resolved by doing site-directed mutagenesis with more than one amino acid substitution at a given site and by ensuring that the substitutions that take place are with amino acids of similar size and characteristics. Two examples of this problem arise in one of the classic papers on mutagenesis of the ligand-binding domain of the LDL receptor (Esser et al. 1988). The substitution of Phe_{181} with Tyr has minimal effects on LDL and ß-VLDL binding. However, if one mutates the same amino acid to Gly, the binding of both ligands is severely impaired.

An even more dramatic example of this problem is demonstrated by point mutations at Asp_{206}. When Esser et al. (Esser et al. 1988) mutated this amino acid to either Tyr or Asn, both LDL and ß-VLDL binding were almost entirely abolished, but transport of the mutant receptor was only mildly affected. However, in the naturally occurring mutant where this Asp is replaced with Glu, a similar amino acid, the transport is markedly delayed, and there is significant LDL and ß-VLDL binding! (Fourie 1988, AM Fourie, GA Coetzee and D.R. van der Westhuyzen, personal communication).

Another problem with these experiments is that the behaviour of mutant receptors has not been studied in hepatocytes, or in other polarised cell types. In vitro investigation of the LDL-receptor structure-function relationships have been almost entirely carried out in skin fibroblasts, COS cells and CHO cells, none of which are polarised. The cell type is almost certain to affect the control of LDL-receptor synthesis (Hep G2 and fibroblasts respond differently to up- and down-regulation (Tam et al. 1991); the rate of LDL-receptor processing from the precursor to the mature form is much slower in COS cells (Esser et al. 1988) than in fibroblasts (Tolleschaug et al. 1982), and the degradation rate of receptors is faster in HepG2 cells than in fibroblasts (D Hendricks, GA Coetzee and DR van der Westhuyzen, personal communication).

In spite of these problems, two classic papers from the Dallas group (Esser et al. 1988, Russell et al. 1989) have made a major contribution to the understanding of the structure-function relationships in the ligand-binding domain of the LDL receptor and will be reviewed in some depth. Plasmids containing the LDL-receptor cDNA were engineered with various amino acid substitutions and deletions and the binding at 4°C of antibody, LDL and ß-VLDL to cells expressing normal and mutant cDNA was determined. Antibody binding was done at saturating concentrations. Ligand binding (LDL and ß-VLDL) was done in most cases at two concentrations: One concentration was below saturating levels, while the other was at saturating ligand concentrations for normal receptors. The binding ability of the receptors was normalised to the number of receptors on the cell surface by dividing the amount of LDL or ß-VLDL bound by the amount of antibody bound (Esser et al. 1988, Russell et al. 1989). The actual affinity of the mutant receptors for the mutant ligands was only measured in a few cases. These studies suggested that repeat 1 was not important for ligand binding (Esser et al. 1988). The only repeat in the ligand binding domain that was crucial for ß-VLDL binding was repeat 5 (Russell et al. 1989). LDL binding was mildly affected by point mutations in or deletion of repeat 2, and severely affected by mutations or deletions in repeats 6,7,3,4 and particularly repeat 5 (Russell et al. 1989). Deletions or mutations in the EGF-precursor homology domain repeats A and/or B did not affect ß-VLDL binding while

mutations in repeat A alone or in both repeats A and B affected LDL binding. Mutations in repeat B alone did not affect LDL binding (Esser et al. 1988). These results implied that the individual repeats operate as semi-independent modules: a mutation in a repeat very often has the same effect as the deletion of that repeat - the implication is that a mutation does not have effects outside its own repeat.

The results of Esser et al. (1988) suggest a particularly interesting phenomenon (Fourie et al. 1988) that was not discussed in that paper: Some of these mutations possibly produce at least 2 distinct functional populations of receptors from one mutant gene. In these experiments the surface number of receptors has been normalised by comparing the antibody binding in the mutant cells to that in normal cells. Any LDL or ß-VLDL binding to the normal and mutant cells was made proportional to the number of surface receptors by dividing by the amount of antibody bound. This corrected binding was then compared to that of the normal receptor and called a percent of normal corrected binding. I will give an example: If the normal cells bind 600 ng IgG-C7/mg cell protein and 100 ng LDL/mg cell protein and the mutant cells bind 300 ng/mg and 10 ng/mg of the respective ligands in parallel experiments, then the normal cells bind 1/6 ng LDL per ng IgG-C7 bound while the mutant cells bind 1/30 ng LDL per ng IgG-C7 bound. Therefore if one corrects for the antibody binding, the mutant receptors bind only 20%

the amount of LDL that the same number of normal receptors would bind. Two models can explain this result. The one possibility is that the affinity of the mutant receptors for LDL is lower than that of the normal receptors. Alternatively, there are two populations of mutant receptors where 20% of the receptors have a normal affinity for LDL and 80% of the receptors do not see LDL at all. A combination of these two models, where there are two or more populations of receptors each with abnormally low but different affinities for LDL, can also explain these results.

In cases where the corrected percentage ligand bound by a mutant receptor is less than 10% of normal at both non-saturating and saturating ligand concentrations (for normal receptors), one can assume that the affinity of the mutant receptors for the ligand has been grossly impaired. Even if there are two receptor populations, the population that recognises ligand is insignificant.

In the scenario where the corrected percentage ligand bound is low at the non-saturating ligand concentration but increases to approach normality at the saturating concentration, one will again assume that the mutant receptors have an impaired affinity for the ligand. When the corrected percentage ligand bound remains at a significant but abnormally low value at both ligand concentrations, one must consider the two-population model. It is difficult to distinguish between one population of receptors with a

decreased affinity for LDL and the 2-population model where one population has a normal affinity for LDL, unless one does binding isotherms at various ligand concentrations as was done in Fig. 8 of the paper by Esser et al. (Fig. 1.2). In this experiment the corrected specific binding has been plotted over a range of ligand concentrations for 4 mutations and compared to the normal binding. The affinities of the mutant receptors have been determined to be approximately normal. If two different receptors have the same affinity for their ligands, one would expect them to show identical curves if the corrected binding values are plotted. The curves will have similar shapes as the affinities are the same and should have the same B_{max} (and thus overlap) since the corrected binding normalises for cell surface receptor expression. Because the affinity of the mutant receptors in this experiment were normal but the proportion of mutant receptors that see a given concentration of LDL as a percentage of antibody binding was less in all cases than the same number of normal receptors (as a percentage of antibody binding), one has to conclude that there are at least two populations of mutant receptors resulting from each of these mutant genes. A naturally occurring mutant that exhibits the same phenomenon is the FH Afrikaner-1 major founder mutation in South Africa (Fourie et al. 1988). This phenomenon should not be that surprising since even the normal receptor does not exist as a single population of identical molecules. There are probably two or more distinctly glycosylated normal receptor sub-populations (Cummings et al. 1983) that might

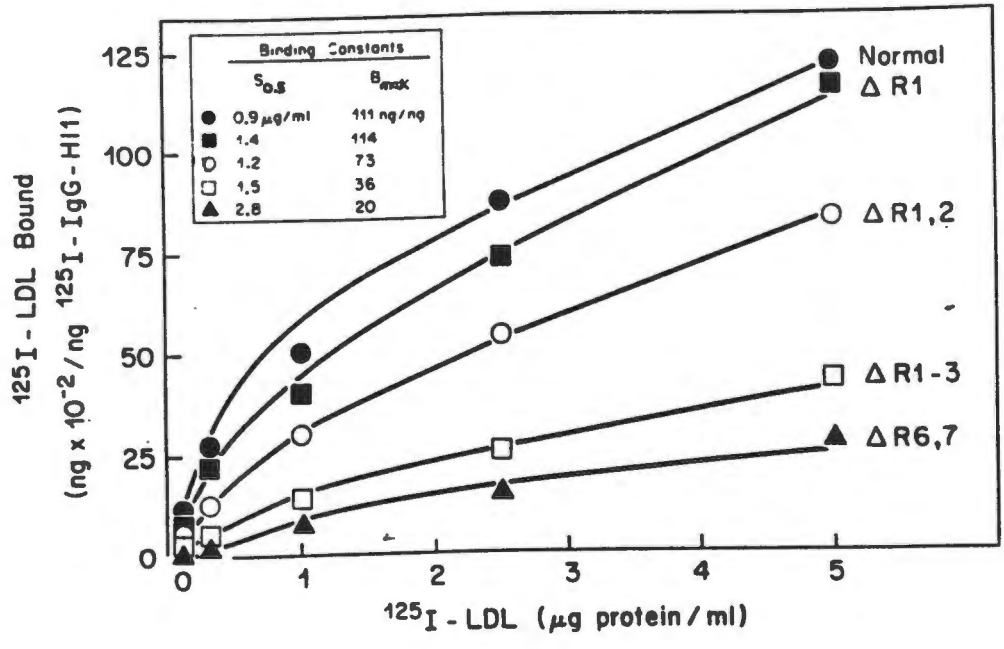


Fig. 1.2: Surface binding of ^{125}I-LDL in COS cells transfected with normal or mutant LDL receptors bearing deletions of ligand binding repeats 1(ΔR1,), 1-2(ΔR1,2), 1-3(ΔR1-3) or 6-7(ΔR6-7) (from Esser et al. 1988). Plasmids encoding the indicated LDL receptors were transfected into COS-M6 cells and assayed for binding of ^{125}I-LDL at the indicated concentrations. The amount (ng/dish) of ^{125}I-LDL bound at each ligand concentration by cells mock-transfected with salmon sperm DNA was subtracted from the value obtained with a given plasmid. This number was corrected for transfection efficiency by dividing it by the amount of ^{125}I-labeled anti-LDL receptor monoclonal antibody bound to cells transfected with the indicated plasmid in the same experiment (2 ug/ml ^{125}I-labeled IgG-HL1 were incubated with cells for 3 h at 4°C). Binding constants (inset) were calculated by the methods of Lineweaver and Burk using a programmable calculator. Reproduced with permission of the authors and American Society for Biochemistry and Cell Biology.

even behave in subtly different fashions. However, in these artificial mutations and in FH Afrikaner-1 (Fourie et al. 1988) we see how one gene product has produced distinct functional populations.

This phenomenon of multiple receptor conformations with different ligand-binding properties produced by a single gene has also been offered as a possible explanation for the non-reciprocal cross-competition of Acetyl-LDL and Oxidised LDL for binding to type I and type II bovine scavenger receptors (Freeman et al. 1991, Krieger 1992).

The effect that O-glycosylation has on receptor binding was demonstrated in a Monensin-resistant CHO cell line that produces LDL receptors that lack selected O-linked sugars (Segushi et al. 1991). About a third of the O-glycosylation of the receptor occurs outside the O-linked sugar domain of the receptor. If this is interfered with, the receptors do not bind ligand normally, suggesting that these sugars play a role in ensuring optimal folding of the receptor.

1.7 MECHANISMS OF MUTATION IN THE LDL-RECEPTOR GENE

Familial hypercholesterolemia with its heterozygote frequency of 1/500 is one of the commonest Mendelian diseases known to man (Goldstein and Brown 1989). Three factors determine the frequency of a genetic disease: Firstly, the susceptibility of a particular gene to mutation; secondly, the extent of heterozygote disadvantage or advantage conferred by

mutations; the third factor includes the chance gene-sampling events like random genetic drift and founder effects that can amplify or reduce a gene's frequency.

The LDL-receptor gene is potentially a highly mutagenic locus (Hobbs et al. 1990). Twenty-five percent of the 45 kB LDL receptor gene has been sequenced and 28 Alu repeats have been found. This is a frequency of about twice that of the average human gene. These Alu repeats are the commonest "middle repetitive" DNA sequences in humans. They are hotspots for recombination events that can occur by at least two mechanisms. Two Alu repeats on the same strand can recombine and cause a deletion when the repeats are in the opposite orientation if the strand forms the approriate stem-loop structure. Alu repeats in the same orientation at different locations but on different chromatids are potential sites for unequal crossing over during meiosis. Theoretically, this will result in one chromosome having an insertion and its homologue having the complementary deletion. This type of Alu-Alu recombination event is the most prevalent . Alu mediated recombination can also involve only a single Alu sequence. Seven of the 8 large deletions in the LDL receptor that have been sequenced involve Alu repeats at one or both of the endpoints (Hobbs et al. 1990).

The other mechanism of mutation that seems to be prevalent in the LDL receptor is the transition of C to T at CpG dinucleotides (Cooper and Krawczak 1990). These transitions

are presumably due to the deamination of 5-methyl-cytosines at these sites. (Most of the methylation of DNA occurs at cytosines in CpG dinucleotides). Once a 5-mC is mutated to a T it is replicated, while if an unmethylated C is deaminated to U it is likely to be repaired to a C by uracil glycosidase (Coulandre et al. 1978). Eight of the 18 known LDL-R point mutations involve C-T transitions at CpG dinucleotides (Hobbs et al. 1990). In the case of the FH Afrikaner-1 mutation the CpG concerned was shown to be methylated (Rideout 1990). This type of hotspot for mutations can give rise to the situation where a particular mutation can occur twice e.g. the FH Zambia (Pro_{664}-Leu) mutation has occurred independently in India and in England and possibly in Norway as well! (King-Underwood et al. 1991).

In certain diseases where the heterozygous disadvantage severely compromises reproductive fitness, the gene frequency is low and is maintained almost exclusively by the mutation rate. However, in FH it is unlikely that there is any significant heterozygote disadvantage since the disease usually only manifests in men after the reproductive age and about 10 years later in women (Thompson et al. 1989). Nobody has suggested a mechanism or evidence for heterozygous advantage in FH. Thus the disease genes are only eliminated from the population by homozygote subjects who usually do not reproduce.

The frequency of FH in most populations is 1/500 but in the Afrikaners, French Canadians and Christian Lebanese it has been amplified by founder effects and/or random genetic drift (Seftel et al. 1980, Moorjani et al. 1989, Khachadurian 1964). The history of the settlement of the Afrikaners in South Africa serves as a classic example of the workings of the founder effect. The majority of the present day population of about 2 million Afrikaners in South Africa are descended from about 2000 settlers of Dutch, German and French origin who arrived in South Africa in the late 17th and early 18th centuries (Theal 1922). These settlers' gene pool evidently had a higher frequency of certain disease alleles and a lower frequency of other alleles compared to their original parent European gene pool. This was the result of chance over- or undersampling of rare alleles. (The odds of such occurrences is inversely proportional to the size of the settler population). This population maintained its altered genetic constitution as it expanded, since until recently Afrikaners generally only married Afrikaners. These altered allelic frequencies were prone to further amplification or reduction by random genetic drift which small populations are prone to. The Afrikaner population expanded far faster than its European counterparts by growing about 1000 fold in 300 years (Dean 1973). Today the frequency of FH has been estimated to be 1/70 (Seftel et al. 1980) and is very largely accounted for by two founder mutations and possibly a third (Leitersdorf et al. 1989b, Kotze et al. 1989). Other diseases that are particularly

prevalent in the Afrikaner population are progressive familial heart block, Huntington's Chorea, porphyria variegata, Gauchers disease, cystic fibrosis and familial colonic polyposis (Botha and Beighton 1980).

South African Jews of Eastern European (Ashkenazi) descent also have a particularly high incidence of FH (> 1/100) (Seftel et al. 1989). The predominant mutation in this population is FH Piscataway (Meiner et al. 1991). The majority of the South African Jewish population are descendents of 40 000 Lithuanian immigrants who settled in the country between 1889 and 1910. In this case it is unlikely that a founder effect could have manifested in three or four generations from a relatively large starting population. Since this mutation also accounts for 35% of FH in Ashkenazi (Jews of Eastern European origin) Israelis, it is more likely that the founder effect occurred in Lithuania. This founder mutation is particularly interesting since evidence of its founder origin are visible in many Ashkenazi Jewish families throught the world (Meiner et al. 1991).

Another South African population that seems to have a particularly high incidence of FH (> 1/100) is the Indian population (Seftel H, Aswat M, personal communication) that is almost entirely descended from 150 000 immigrants who settled in South Africa between 1860 and 1911. The majority of these people were indentured labourers who originated from many different regions on the the South and East Coasts of

India. A minority (less than 30 000) were traders and artisans who paid their own passage to South Africa. This group, known as the "passenger Indians", came from areas in the Gujerat province on the west coast of the subcontinent. About half of these persons came from the Surat and Valsad districts. The other major source of passenger Indians was Kathiowar on the Arabian Sea (Bhana and Brain 1990). The Indian population of South Africa is of diverse cultural and religious origin; the majority (65%) of the population are Hindus (many of the castes are represented) and there are a significant number of Muslims (21%) and Christians (7.5 %) (Lazarus 1972).

1.8 FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

1.8.1 Historical

One of the major roles of Apo B-100 is to serve as the ligand on LDL for its binding to the LDL receptor. The possibility that a binding-defective apo B could be of pathological consequence was strongly suggested by the discovery of a group of hypercholesterolemic patients who manifested delayed clearance of autologous LDL but not of heterologous LDL (Vega and Grundy 1986). Subsequently, LDL from these patients was shown to bind poorly to the LDL receptor (Innerarity et al. 1987). The binding site of apo B was the region most likely to harbour a mutation that would have these consequences. Thus, LDL from these patients and normal controls was examined with respect to its antigenicity to 4 different antibodies whose epitopes are in the putative binding domain

of apo B-100. Three of the antibodies showed no difference in binding but the antibody MB47 showed preferential binding to the LDL from the patients (Weisgraber et al. 1988). A large region of the apo B-100 DNA from the patients was sequenced and a single point mutation was detected at position 3500. This resulted in the codon for arginine being replaced by glutamine (Soria et al. 1989). Although the functional consequences of this mutation have not been confirmed by the classical techniques of site directed mutagenesis, there is convincing epidemiological and physical chemistry evidence to suggest that the Arg-Gln mutation is responsible for impaired apo B-100 binding in these subjects (Innerarity et al. 1990).

The prevalence of this disorder has been estimated to be around 1/500 in North American and Western European populations (Innerarity et al. 1990), which would make it one of the most common Mendelian disorders. Since this mutation is a C to T transition at a CpG dinucleotide and was possibly caused by deamination of a methyl-cytosine, the mutation could have occurred more than once. Ten different Apo B allelic markers were analysed in 8 subjects with the Apo B-3500 mutation in order to determine the haplotypes of the mutant alleles. The mutant alleles of 7 of the subjects were the same. The 8th subject's mutant allele was concordant with those from the other subjects for 9 of the markers. The only difference in this subject was that he had 46 instead of 48 repeats in the 3' VNTR of the apo B gene. This difference

could have arisen either from slippage or recombination. A further 6 subjects with this mutation were found to have mutant genes with the same haplotype as the 7 subjects that were initially examined. These findings suggest that the apo B-3500 mutation is a founder mutation (Ludwig and McCarthy 1990). One should thus not expect this mutation to be equally prevalent in all populations. Indeed, this mutation was not detected in more than 500 Finnish hypercholesterolemic subjects suggesting that this is not a common cause of raised plasma cholesterol in this population (Hamalainen et al. 1990).

1.8.2 Clinical

There has been some controversy as to the severity of the hypercholesterolemic and atherogenic consequences FDB compared to FH. This problem was initially exposed by the difference between a moderately hypercholesterolemic North American group of FDB patients (Innerarity et al. 1990) and an English and Scandinavian cohort (Tybjaerg-Hansen et al. 1990) who were practically indistinguishable from FH heterozygotes. Innerarity et al. (1990) suggested that there was a danger of equating the severity of this disease to FH if one looks for it in an FH-like population from the start. It was possible that there were less severely affected individuals who were not being detected because they did not have an FH phenotype. This debate has not been entirely resolved but it would seem that FDB is often clinically indistinguishable from FH with respect to its degree of

hypercholesterolemia and frequency of xanthomata, arcus cornealis and premature atherosclerosis (Rauh et al. 1992). Conflicting reports exist as to the effect of drugs that upregulate LDL-receptor function on the hypercholesterolemia resulting from FDB. Although an initial report suggested that FDB was perhaps refractory to such therapy (Corsini et al. 1991), Maher et al. (1991) showed very similar responses to cholystyramine and/or simvastatin in 11 FDB subjects and FH controls matched for age, apo E phenotype and starting LDL-cholesterol concentrations.

Dunning et al. (1991) recently presented evidence that this mutation at codon 3500 might not be the only apo B mutation that causes hypercholesterolemia. They examined the apo B gene sequence encoding amino acids 3130-3630 in 8 hypercholesterolemic individuals who were selected either on the basis of delayed clearance of autologous LDL or impaired binding of their LDL to normal LDL receptors. Since no sequence abnormalities were detected in the area of the gene examined, they concluded that mutations outside the putative binding domain of the apo B gene were affecting its interaction with the LDL receptor. Thus, there are possibly mutations in apo B-100 other than that at codon 3500 that can cause hypercholesterolemia.

1.9 THE AIMS OF THIS THESIS

The clinical picture of familial hypercholesterolemia can result from mutations at either the LDL-receptor gene locus

or at codon 3500 in the apo B gene (Rauh et al. 1992). Thus, at the risk of being pedantic, a patient with clinically defined familial hypercholesterolemia should be provisionally labelled as having monogenic hypercholesterolemia until the defect has been localised to either the LDL receptor or apo B genes. The Afrikaner (Seftel et al. 1980), Jewish (Seftel et al. 1989) and Indian populations in South Africa (Seftel H, Asvat M, personal communication) have particularly high frequencies of clinically defined monogenic hypercholesterolemia. Founder LDL-receptor mutations have been shown to account for most of this disease in the Jewish and Afrikaner groups (Leitersdorf et al. 1989, Meiner et al. 1991).

The nature of the mutations that result in this disease in South African Indians was unknown. Skin fibroblasts were received from the 4 clinically diagnosed FH homozygotes that attended the various lipid clinics in the country, so that their mutations could be studied. The behaviour of the mutant proteins was initially investigated in order to allow us to predict the site of the mutations from the type of defects.

These mutations were also examined at the DNA level. Knowledge of the exact nature of the mutations is a vital part of the puzzle that allows one to consider structure/function relationships. The definition of a mutation at the DNA level would also allow one to screen

likely FH patients in the Indian population. The possibility existed that familial hypercholesterolemia in this group might have been caused by one or two predominant mutations. If this were the case, the discovery of these mutations would allow for rapid and confident diagnosis of LDL-receptor mutations in Indians with definite or possible clinical monogenic hypercholesterolemia.

Although FH does not seem to be common in the South African "Black" population (Marais and Berger 1986), two different FH homozygotes had been discovered (van Wingerdin 1981, Leitersdorf et al. 1988). The nature of the mutations that resulted in low cell-surface receptor-numbers in the one subject, JL, was not known (Coetzee et al. 1985). Preliminary investigations into the behaviour of the mutant LDL-receptor proteins were undertaken in order to direct us to the possible nature of the gene defect(s) in this subject.

The detection of point mutations can be very laborious if one relies entirely on sequencing DNA. Orita and co-workers (1989a, 1989b) described a rapid and easy method to detect point mutations by comparing the electrophoretic mobility on non-denaturing gels of single-stranded DNA from mutant and wild-type genes. The sensitivity and utility of this so-called single-strand conformational polymorphism (SSCP) technique was investigated.

Although founder LDL-receptor mutations have been shown to account for the majority of clinical FH in Afrikaners and Jews, a significant number of patients in these groups remained that do not have these mutations (Graadt van Roggen et al. 1991, Meiner et al. 1991). Both of these populations and the so-called Coloured population (of mixed European, Indonesian and native African descent) have European ancestry. Since the familial defective apo B-100 mutation has a prevalence of 1/500 in European and North American populations (Innerarity et al. 1990, Schuster et al. 1990, Tjaeberg-Hansen et al. 1990), these populations were screened for this mutation.

The FDB mutation seems to have arisen once on an ancestral chromosome (Ludwig and McCarthy 1990) and although quite a number of groups have identified it in populations of presumably European origin, the incidence of this mutation had not been investigated much in non-European/North American or non-Causasian populations (Ludwig and McCarthy 1990). The availability of a number of hypercholesterolemic Indian patients from lipid clinics around South Africa allowed us to look for FDB in such a population.

In summary, the main aim of this thesis was to try and gain an understanding of the LDL-receptor mutations that cause familial hypercholesterolemia in the South African Indian population. An additional aim was to determine whether or

not the FDB mutation was a common cause of monogenic hypercholesterolemia in the Afrikaners, Jews, so-called Coloureds and Indians in South Africa.

CHAPTER 2

SECTION A: GENERAL METHODS

2A.1 Patients

Blood and fibroblast specimens from the patients used in these studies were kindly provided by physicians at Lipid Clinics in Johannesburg (Prof. H. Seftel, Dr D. Raal), Cape Town (Dr A.D. Marais), Durban (Dr I. Jialal, Prof. G.M. Berger) and Potchefstroom (Prof. E. Vorster).

2A.2 DNA isolation from blood or tissue culture cells

DNA isolation was performed either by conventional Proteinase K treatment of cells followed by phenol/chloroform extraction (blood and tissue culture cells) (Method 1) or by using a rapid method (blood only) described by Talmud et al. (1991) (Method 2).

2A.2.1 METHOD 1 (Strauss 1987)

Fibroblasts (about 10^6 cells) were trypsinised and resuspended in ice-cold phosphate-buffered saline (PBS). The cells were then pelleted by centrifuging for 5 minutes at 500 g, resuspended in PBS and repelleted. Fibroblasts or buffy layers were resuspended in 300 µl of digestion buffer containing 100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS and 0.1 mg/ml Proteinase K (Boehringer Mannheim, Mannheim, Germany) and placed in a 1.5 ml microfuge tube. This digestion buffer was freshly prepared. The cells were digested overnight at 50°C. After digestion the samples were extracted with 300 µl

phenol/chloroform/isoamylalcohol (25:24:1). The aqueous phase was then re-extracted with 300 µl chloroform/isoamylalcohol (24:1). The aqueous phase was transferred to a new microfuge tube to which 150 µl 7.5 M ammonium acetate and 600 µl 100% ethanol was added. The DNA was precipitated by centrifugation at 12 000 g for 2 minutes. The pellet was rinsed with 70% ethanol, air-dried in a Speedvac (Savant Instruments Inc.) and resuspended in H_2O or TE pH 8.0 (Strauss, 1987).

2A.2.2 METHOD 2 (Talmud et al. 1991)

DNA from whole blood for PCR amplification was frequently prepared by this rapid procedure: 100 µl whole blood anticoagulated with EDTA (1.5 mg/ml) was mixed with 400 µl freshly prepared 0.17 M ammonium chloride and left for 20 min at room temperature. This was then washed three times by first centrifuging the contents at 12 000 g for 30 sec, then adding 1 ml 0.9% NaCl to the pellet, vortexing and re-centrifuging. The pellet left after centrifuging for 30 sec after the third wash was resuspended in 200 µl 0.05 M NaOH and boiled for 10 min with occasional agitation. This was then neutralized with 25 µl 1M Tris pH 8.0. Three to five µl of this preparation was used per PCR (Talmud et al. 1991).

2A.3 RNA Isolation

RNA isolation from tissue culture cells was performed essentially as described by Chomczynskj and Sacchi (1987). Fibroblasts were seeded at 120 000 cells per 10 cm Petri dish

and grown in Dulbecco's MEM (DMEM) with 10% fetal calf serum for 5 days with a medium change on day 3. On day 5 the medium was changed to DMEM containing lipoprotein deficient serum (LPDS) (5 μg/protein/ml). The cells were then harvested on day 6. The dishes were washed three times with ice-cold phosphate buffered saline. Six hundred microlitres of a digestion solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol was added to each dish. The digested cells were "scraped" from their dishes and collected in 1.5 ml microfuge tubes to which 60 μl sodium acetate pH 4.0 was added and the contents were mixed by inversion. Then 600 μl phenol (water-saturated) and 120 μl chloroform/isoamylalcohol (49:1) were sequentially added and mixed in the tubes by inversion. The tubes were left on ice for 15 minutes and then centrifuged at 4°C in a microfuge (12 000 g) for 20 minutes. The aqueous phase was then transferred to a fresh tube to which 600 μl cold isopropanol was added. The tube was placed at -20°C for at least 1 hour. The tube was then centrifuged at 12 000 g for 20 minutes at 4°C to pellet the RNA. The pellet was air-dried and resuspended in 600 μl of the digestion buffer that was initially used and the RNA was precipitated again with 300 μl isopropanol at -20°C for at least 1 hour. The RNA was pelleted by centrifugation at 12 000 g for 10 minutes at 4°C. The pellet was washed once with 75% ethanol, sedimented and air-dried. Care was taken not to over-dry the pellet. The pellet was then dissolved in H_2O. RNA preparation was always carried out with gloves on.

In some cases diethylpyrocarbonate (DEPC)-treated solutions, microfuge tubes and pipette tips were used to prevent RNAse activity. DEPC treatment was done according to Gilman (1987): 0.2 ml of diethylpyrocarbonate (DEPC) was mixed into 100 ml of the solution to be treated. This was autoclaved to inactivate the remaining DEPC.

2A.4 cDNA Synthesis

cDNA first strand synthesis was carried out using the Geneamp^{R} RNA PCR kit and protocol (Perkin Elmer Cetus, Norwalk, CT) with the random hexamers provided. A 20 μl mix was made containing the RNA, 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM of each dNTP, 1 u/μl RNAse inhibitor, 2.5 μM random hexamers and 2.5 u/ml reverse transcriptase. This mix was overlaid with mineral oil and placed at room temperature for 10 minutes. The tubes were then incubated at 42°C for 15 minutes, 99°C for 5 minutes and placed on ice for 5 minutes. Three to five μl of this reaction was used in subsequent PCR reactions that generated double-stranded cDNA.

2A.5 Kinasing of Oligonucleotides

Oligonucleotides were kinased in a final volume of 10-50 μl containing 10-50 pmol of oligonucleotide, 140 μCi of [τ-^{32}P]-ATP (made using the gammaprep kit (Promega, Madison WI)), 8μ T4 polynucleotide kinase (Promega) and 1/10 volume of 10x polynucleotide kinase buffer (Promega). This was incubated at 37°C for at least 30 minutes.

2A.6 Isolation of DNA fragments from Gels:

2A.6.1 Polyacrylamide Gels

DNA was electrophoresed on polyacrylamide gels of the appropriate percentage and the gel was stained in ethidium bromide (Sambrook et al. 1989). The bands were visualised with a UV lamp and the bands were cut out using a new surgical blade. Each gel slice was broken up with a sterile pipette tip in a 1.5 ml microfuge tube to which 350 µl of a solution of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8, was then added. The tubes were incubated at 37°C in a rotary mixer overnight. The samples were centrifuged at 12 000 g for 1 minute to pellet the acrylamide. The solution was then transferred to a new microfuge tube and recentrifuged at 12 000 g for 1 minute. The solution was transferred to a fresh microfuge tube and the DNA was precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes 100% ethanol and then placing it at -70°C for at least 1 hour. The DNA was pelleted by centrifugation for 30 minutes at 12 000 g at 4°C and was washed with 70% ethanol. The sample was recentrifuged at 12 000 g for 20 minutes, the ethanol was removed and the DNA was vacuum dried in a SpeedVac (Savant Instruments Inc.). The DNA was resuspended in an appropriate volume of TE pH 8.0 or H_2O.

2A.6.2 Agarose Gels

DNA electrophoresed on agarose gels (Sambrook et al. 1989) was also visualised on a UV box after ethidium bromide

staining and the correct bands were cut out. DNA was eluted from the agarose slices using Geneclean II (Bio 101, La Jolla, CA, USA).

2A.7 DNA Sequencing

DNA sequences were determined by one of three different methods. Direct sequencing of PCR products was done either with (2A.7.1) the Maxam and Gilbert system (NEM^{R} DU PONT) or (2A.7.2) with a modification of the Sanger method using a thermal cycler, the fmol^{TM} DNA Sequencing system (Promega, Madison, WI). (2A.7.3) DNA sequencing of cloned material was done with the Taq Track^{TM} sequencing system, a modification of the Sanger method that uses Taq polymerase as the extension enzyme.

2A.7.1 Maxam and Gilbert sequencing:

Maxam and Gilbert sequencing (Maxam and Gilbert 1980) was carried out essentially as described in the kit's booklet (NEN, DUPONT). The following changes were made to the prescribed method. PCR reactions were performed using one primer that had been kinased with [γ-^{32}P]-ATP and one unlabelled primer. The PCR product was electrophoresed on a 6% polyacrylamide gel, and the correct bands were eluted from the gel (see section 2A.6.1). G modification with DMS was performed for 4 minutes while the other three modification reactions (A+G, T+C and C) were performed for 10 minutes. After modification of the appropriate bases the DNA was precipitated with tRNA, ammonium acetate and ethanol

according to the protocol but the pellet was washed three times (as opposed to twice) with cold 75% ethanol prior to piperidine cleavage.

2A.7.2 Direct sequencing of PCR products with the fmol kit (Promega, Madison, WI)

Direct sequencing of PCR products was also performed using the fmol sequencing kit (Promega, Madison, WI). The PCR products were electrophoresed on 1% agarose gels and the appropriate bands were cut out of the gel. DNA was extracted from the gel using the Geneclean II kit (BIO 101 Inc, La Jolla, CA). Sequencing was performed using primers kinased with [γ-^{32}P]-ATP (see section 2A.5) according to the protocol. This method of sequencing is a modification of Sanger di-deoxy sequencing (Sanger et al. 1977) that uses Taq Polymerase on a thermal cycler to accomplish many cycles of sequencing. The linear amplification of the resultant sequence reduces the amount of template needed. The high temperatures used have certain theoretical advantages: alkali denaturation of double-stranded DNA is not needed; there is reduced re-annealing of double stranded templates; there is increased stringency of primer-template annealing and the secondary structure of the DNA templates is reduced. We sequenced exactly according to the kit's protocol. We initially denatured our sample at 93.5°C for 2 minutes before sequencing with 42 cycles of 93.5°C for 35 seconds, 62.5°C for 30 seconds and 72°C for 30 seconds. After the cycles terminated we employed the following procedure to extract the

mineral oil that tended to cause smears on the gels if not removed completely. Ninety-five μl H_2O were added to each tube to which 300 μl of the following stop buffer containing 0.01 M Tris pH 7.5, 4 mM EDTA pH 8.0, 258 mM sodium acetate pH 5.2, 67 μg/ml yeast tRNA, was added. The PCR microfuge tube was centrifuged for 30 seconds at 12 000 g and the contents were transferred to a 1.5 ml microfuge tube, extracted with 400 μl phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with 940 μl 100% ethanol. After washing the pellets with 70% ethanol and drying, the DNA was resuspended in 3-8 μl of loading dye from the kit.

2A.7.3 Sequencing of cloned material with Taq Track Kit (Promega, WI, Madison)

Sequencing of cloned DNA was performed with the Taq Track Kit (Promega WI, Madison), a variation of the Sanger (Sanger et al. 1977) sequencing system. Oligonucleotides were kinased with [γ-^{32}P]ATP (see section 2A.5) and the plasmid template was alkali denatured, ethanol precipitated and sequenced exactly as described in the protocol.

Sequences generated by all of the above methods were denatured at 95°C (Taq Track, Maxam-Gilbert) or 75°C (fmol Kit) prior to electrophoresis on 6% or 8% acrylamide, 7 M urea sequencing gels (Slatko and Albright 1989).

Table 2A.1: Oligonucleotides, reaction conditions and gene regions amplified by PCR

Oligo-nucleotide Code	Gene and gene region amplified	Amplification conditions	Oligonucleotide sequences (5'-3')	+/-BSA
	LDL-Receptor cDNA			
AR12 AR13	Exons 1-4	1x(94°C for 10 min, Add Taq, 68°C for 12 min)	ATA GGA TCC AAA TTG CGC TGG ACC GTC GCC TTG C ATA GCA TGC TCG TCA GGG CGA CAG GTG GCC ACA G	-
AR14 AR15	Exons 5-8	35x(93°C for 1.5 min + 68°C for 5 min)	ATA GGA TCC GAC AAA TCT GAC GAG GAA AAC TGC G ATA GCA TGC GTG AAG AAG AGG TAG GCG ATG GAG C	-
AR16 AR17	Exons 9-12	1x68° for 10 minutes	ATA GGA TCC CAC ACG AAG GCC TGC AAG GCT GTG G ATA GCA TGC TGA TAT CTG TCC AAA ATA CTT TGT C	-
AR6 AR7	Exons 13-15		CGC GGA TCC CCC CTT CTC CTT GGC CGT CTT TGA G CGC GGA TCC CCT CTG CCA GCA ACG TCG CCC AGA G	-
AR8 AR9	Exons 16-18		CGC GGA TCC GAG ATA GTG ACA ATG TCT CAC CAA G CGC GGA TCC CAG GAA GGG TTC TGG GCA GGG GCG G	-
G13 G14	Exons 1-3	(98°x5min add Taq) 37x (93°C for 35sec, 62.5° x 3 min)	TAT GTC GAC ACT GCC TGG CAG AGG C GAT CAA GCT TCC CAT CGT GGC AGC GA	+
G25 G26	Exon 4	1x93° for 35 sec + 62.5° for 6 min	ATA TCT AGA CGA GCA AGG CTG TCC CC GAG GAA TTC GTC AGG GCG ACA GG	+
G27 G28	Exons 5 and 6 + ½ of 7		ATA GAA TTC CAG CTG GCG CTG TGA TG AAA GTC GAC TCG TAG CCG ATC TTA AGG	+
LDLR-P2	LDL Receptor DNA LDL-R promoter	35x(93°C for 35sec, 59°C for 2min, 72°C for 2min)	TTC TCT GGG ACA GGT CAG ACC AGT	+
G8		1x(93°C for 35sec, 59°C for 2min, 72°C for 10min)	ACC TGC TGT GTC CTA GCT GG	
3.1 5	Exon 3	40x(93°C for 1 min, 53°C for 1 min, 72°C for 1min)	ACT TAC GAC AGC CTT GCT CG CTG TCT CTT CTG TAG TGT CT	-
G31 4.3	Exon 4 (5' end)	1x(93°C for 1 min, 55°C for 1 min, 72°C for 1min)	CAT CCA TCC CTG CAG CCC CC GGG GTC GTT GTC GCA GGC CC	-
541 542	Exon 13, intron 13 and Exon 14	35x(93°C for 35 sec, 60°C for 3 min) 1x(93°C x 35 sec, 60°C for 6 min)	AAT GTC GAC GTC ATC TTC CTT GCT GCC TGT TAT GTC CAG AAA CAA GGC GTG TGC CAC	+
	Taq 1 RFLP (intron 4)	30x(93°C for 1 min, 65°C for 4 min)	ATC TGA CGA GGA AAA CTG CGG GGC CAC AGC TGG AAA ACA GGA	-
	Stu 1 RFLP (Exon 8)		ATC GAT GAG TGT CAG GAT CC CCC GCC GCC TTC CCG TGC TGA	-
	Ava II RFLP (Exon 13)	35x(93°C for 1 min, 55°C for 2 min, 72 x 2 min)	AGT GCC AAG CGC CTC ACA GG CCT CTC ACA CCA GTT CAC TC	-
	Nco 1 RFLP (Exon 18)		CAC CTA GTG CTT CCA CTT CTA TCC CGT CAA ACG ATC CAG ACT	-
	LDL-R exon 4			
D1 D2		35x(93°C for 35 sec, 60°C for 3 min) 1x(93°C for 35 sec, 60°C for 6 min)	CCC CAG CTG TGG GCC TGC GAC AAC G CGC CCA TAC CGC AGT TTT CC	-
G23 G24	Apo E gene for genotyping	95°C for 5 min, Add Taq; 35x(94.5°C for 25 sec, 60°C for 1 min) (include 5% DMSO in PCR mix)	TAA GCT TGG CAC GGC TGT CCA A GGA ACA GAA TTC GCC CCG GCC TGG TAC AC	-
	Apo B gene for diagnosing 3500 mutation			
B1 B2		40x(93°C for 35 sec, 56°C for 1 min, 72°C for 4 min)	CCA ACA CTT ACT TGA ATT CCA AGA GCA CCC CTG TGC TCC CAG AGG GAA TAT ATG CGT TGG	+

2A.8 PCR

Each 50 µl PCR reaction mix contained genomic DNA or cDNA (1st strand), 2.5 u Sequencing grade Taq polymerase (5u for amplification of cDNA) (Promega, Madison, WI, USA), 5 ul of 10x Taq polymerase buffer (Promega), 100 µM of each dNTP, 20 pmol of each primer and 500 ng genomic DNA or 3 to 5 µl of rapid preparation DNA. Acetylated BSA (100 µg/ml) (Promega) was added to some of the reactions. The exact conditions and oligonucleotides used in each PCR reaction are shown in Table 2A.1. Oligonucleotides were synthesised on an AUTOGEN^{TM} machine (Milligene Corporation, U.S.A.)

2A.9 Diagnosis of Mutations by PCR with/without restriction enzyme analysis

2A.9.1 FH Zambia (Pro_{664} to Leu)

This mutation was detected according to the method of Soutar et al. (1989). Exons 13 and 14 were amplified using oligonucleotides 541 and 542 (kindly provided by Dr A Soutar, MRC Lipoprotein Team, Hammersmith Hospital, London, UK) as described in Table 2A.1. After analysing 15 µl of the product by electrophoresis in 1% agarose, the remaining 35 µl was digested overnight at 37°C with 10 U Pst I (Boehringer Mannheim, Mannheim, Germany) in a final volume of 40 µl containing 4 µl 10x H buffer (Boehringer Manhheim). This was then analysed by electrophoresis on a 6% acrylamide gel. This mutation creates a Pst I cutting site in exon 14 (see Fig. 5.1).

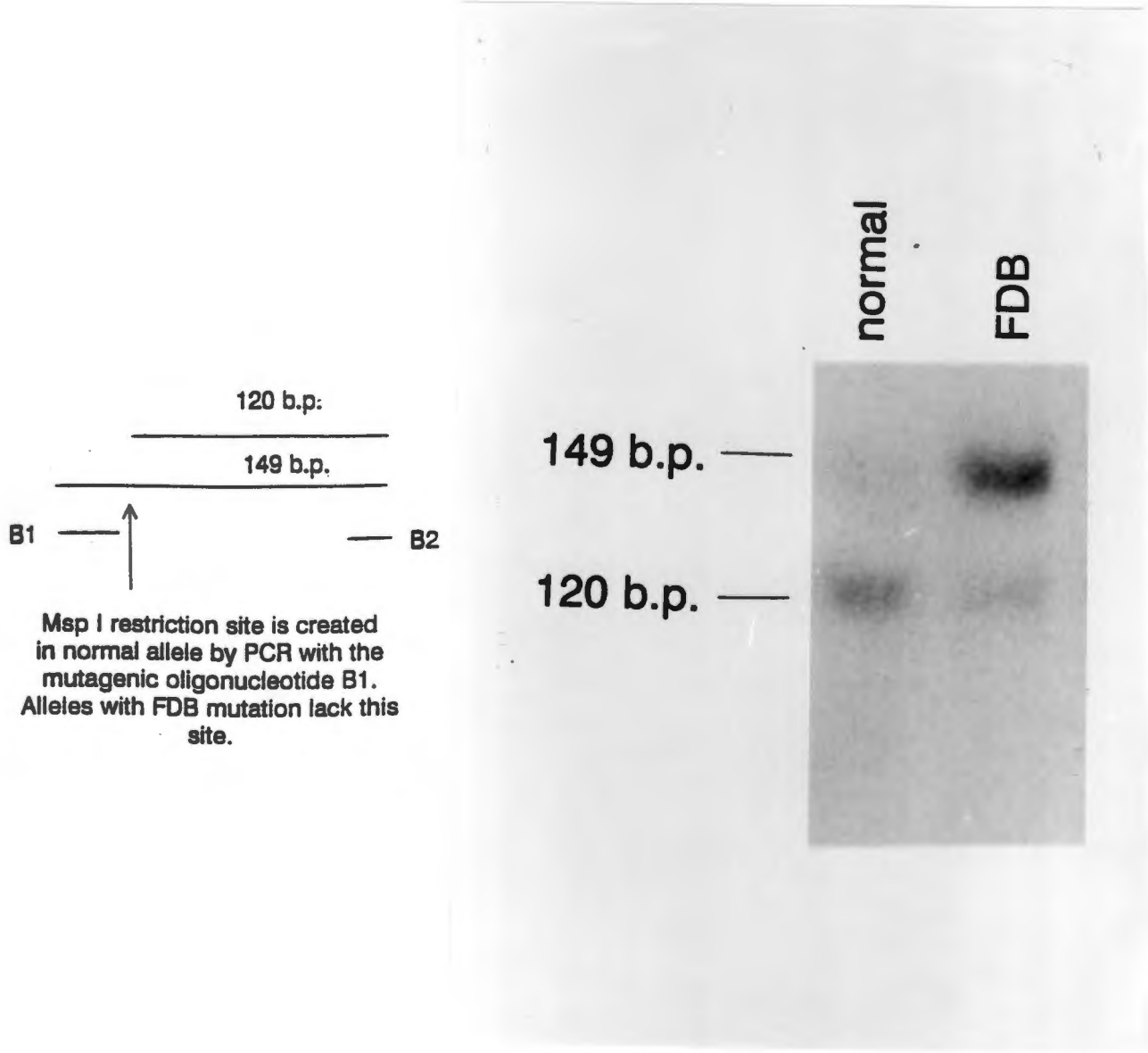


Fig. 2A.1: Assay for the FDB mutation. Genomic DNA was amplified in a PCR reaction containing oligonucleotides B1 and B2 (see table 2A.1) and 2 μCi [α-^{32}P] dCTP. PCR products were digested with 20 u Msp I and were electrophoresed on 12% Acrylamide. The gels were dried and exposed to X-ray film overnight.

2A.9.2 Familial Defective Apolipoprotein B-100 (Arg_{3500} - Gln)

This mutation was detected exactly according to the method described by Hansen et al.(1991). This assay uses an oligonucleotide (B1) with a single base pair mismatch 2 bp from its 3' end that introduces an Msp I cleavage site in the PCR product (generated with B2, see Table 2A.1) of the normal allele. The normal alleles are digested by this enzyme but the mutant alleles are not since they do not have the recognition sequence (Fig. 2A.1). It was difficult to achieve 100% digestion of our PCR products even if they were phenol/chloroform extracted and ethanol precipitated prior to digestion with 20 u MspI. A subject was considered normal if the digestion was clearly more than 50%. The diagnosis of FDB in subjects where there was obviously abnormally little digestion was confirmed by repetition of the experiment or by digestion of a small amount of [α-^{32}P]dCTP-labelled normal PCR product in the same tube as unlabelled suspect DNA. The suspect DNA was confirmed to be FDB since there was clearly more digestion of normal DNA as judged by autoradiography of the sample run on a 12% acrylamide gel than of the mutant sample as judged by ethidium bromide staining of our gel. (The converse situation was also used to confirm the mutation).

2A.9.3 LDL-receptor Haplotypes

The LDL-receptor RFLPs were determined using PCR amplification with oligonucleotides flanking the variable

restriction sites for Taq 1, Stu 1, Hinc II, Ava II and Nco 1 (Leitersdorf and Hobbs 1989, Leitersdorf et al. 1989a, Meiner et al. 1992) (see Table 2A.1). Some of the haplotyping was done in the laboratory of Dr E. Leitersdorf (Lipid Research Laboratory, Haddassah University Hospital, Jerusalem, Israel) and some was done by me.

2A.9.4 Diagnosis of 'D' mutation in exon 4 (Glu_{119}-Lys)

This mutation eliminates a Mnl I site in exon 4. The 5' end of exon 4 was amplified using oligonucleotides G31 and 4.3 as described in Table 2A.1. Each PCR mix was spiked with 2 μCi [α-^{32}P] dCTP. Four microlitres of each sample were digested in a final volume of 30 μl containing 3 μl Buffer 2 (New England Biolabs, Beverly, MA), 3 μl acetylated BSA (1 mg/ml, Promega), and 4 u Mnl I (New England Biolabs). The digested products were electrophoresed on 12% Polyacrylamide gels. The gel was dried and exposed to X-ray film overnight (See Fig. 4.12).

2A.9.5 Diagnosis of Lithuanian Jewish mutation, FH Piscataway (deletion of Gly_{197})

Part of exon four spanning Gly_{197} was PCR-amplified with oligonucleotides D1 and D2 (Table 2A.1). The heteroduplexes formed between amplification products of the normal and mutant alleles run with a slower mobility than homoduplexes on 6% polyacrylamide gels (Meiner 1991) (Fig. 2A.2).

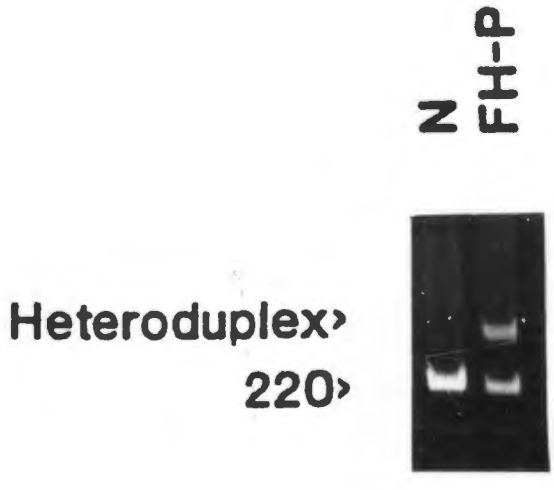


Fig. 2A.2: Diagnosis of the FH Piscataway LDL-receptor mutation. Genomic DNA was amplified by PCR using oligonucleotides D1 and D2 that span part of exon 4 of the LDL-receptor gene (see table 2A.1). The PCR products were electrophoresed on 6% Acrylamide and the heteroduplexes formed were characteristic in heterozygotes of this mutation, a 3 b.p. deletion of Gly_{197}.

2A.9.6 Detection of FH Afrikaner-1 and FH Afrikaner-3 LDL-receptor mutations

These mutations, both in exon 4, were screened for by PCR-amplification of a 220 b.p. region of that exon using oligonucleotides D1 and D2 (see PCR Table 2A.1) and by subsequent restriction digestion with Dde 1, the site created by the FH Afrikaner-1 mutation, or Mbo II, the site abolished by the FH Afrikaner-3 allele. These assays were performed essentially as described (Graadt van Roggen et al. 1991). Each PCR reaction was spiked with 2-4 μCi [α^{32}P]-dCTP. Ten microlitres of the amplified product were digested overnight at 37°C with Dde I (Promega 4u) or Mbo II (New England Biolabs 2.5u) without the addition of any additional buffers to the PCR mix. The digested products were electrophoresed on 12% Polyacrylamide (Dde I) or 10% Polyacrylamide (or 2 % Agarose) (Mbo II). Dde 1 digests normal alleles into two fragments of 130 and 90 b.p. and FH-Afrikaner 1 alleles into 130, 64 and 26 b.p. (Fig. 2A.3). Mbo II digests normal alleles into 171 and 49 b.p. fragments and the FH Afrikaner-3 alleles are resistant to digestion (Fig. 2A.4).

2A.9.7 Detection of FH Afrikaner-2 mutation (and familial defective Apo B-100)

This method was devised by E. Langenhoven and M. Kotze (Department of Human Genetics, Faculty of Medicine, University of Stellenbosch) and was kindly conveyed to us. The ARMS (Amplification Refractory Mutation System) PCR method is used to detect the FH Afrikaner-2 mutation while

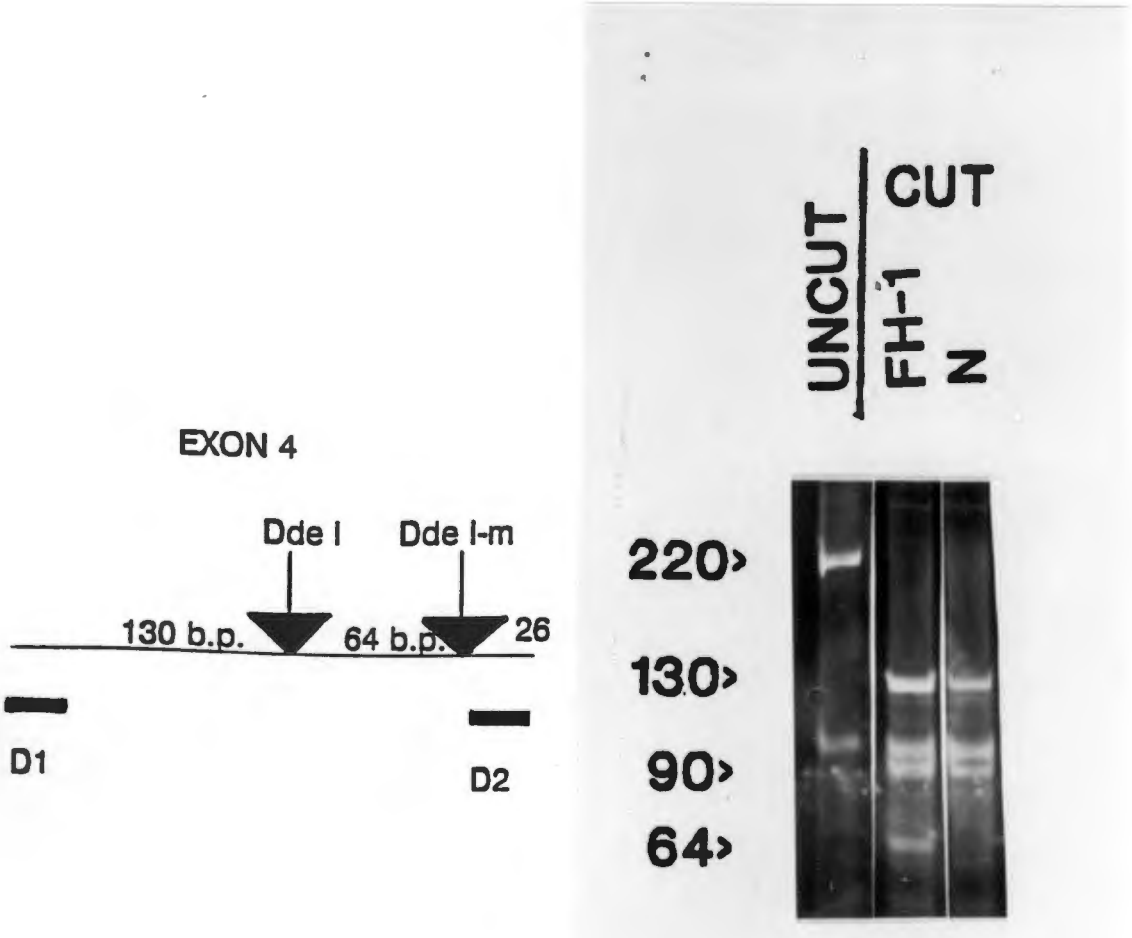


Fig. 2A.3: Assay for diagnosis of the FH Afrikaner-1 mutation. Genomic DNA was amplified by PCR using oligonucleotides D1 and D2 that span part of exon 4 of the LDL-receptor gene (see Table 2A.1). PCR products were digested overnight at 37°C with 4 u Dde I and then electrophoresed on 12% Acrylamide. This mutation creates a new Dde I site (Dde I-m) that results in the formation of a 64 b.p. fragment. (The band just above 90 b.p. in the uncut sample that persists in the cut samples is probably a primer dimer-type PCR artefact that is unrelated to the LDL-R fragment concerned as it is not digested by Dde I.)

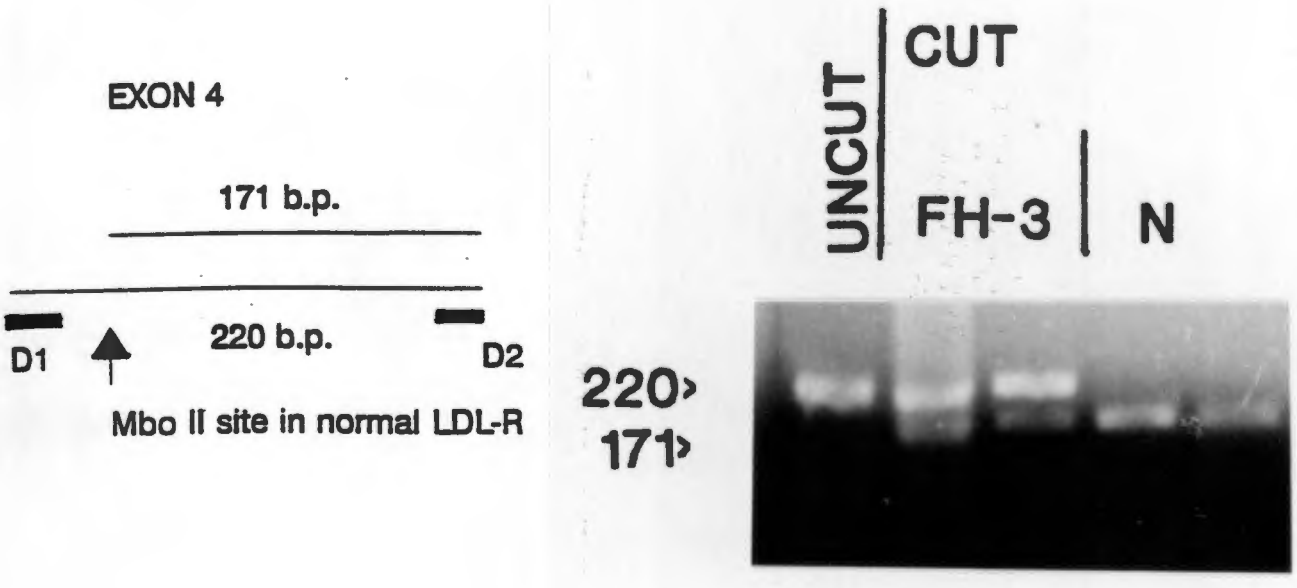


Fig. 2A.4: Assay for diagnosis of the FH-Afrikaner-3 mutation. Genomic DNA was amplified by PCR using oligonucleotides D1 and D2 that span part of exon 4 of the LDL-receptor gene (see table 2A.1). The PCR products were digested overnight at 37°C with 2.5 u Mbo II and the products were electrophoresed on 2% Agarose. This mutation abolishes an Mbo II site and the persistence of a 220 b.p. fragment after Mbo II digestion is diagnostic for this mutation.

the same PCR tube also contains oligonucleotides that amplify the apo B gene which serves as an internal control for the PCR. The apo B PCR product can be used for detection of FDB by differential oligonucleotide hybridisation (Tybjaerg-Hansen et al. 1990). Each sample is placed into two different PCR mixes for analysis by ARMS. Each mix contained the oligonucleotides AB-1 (5'-GGAGCAGTTGACCACAAGCTTAGC-3') and AB-2(5'-CAGGGTGGCTTTGCTTGTATGTTC-3') that amplify a 345 b.p. region of the apo B gene. The oligonucleotide 9.5 (5'-GCTCACCTGCAGATCATTCTCTGGG-3') was the common distal primer for amplification of exon 9 of the LDL receptor and was added to both tubes that contained either one of the allele specific oligonucleotides 9.3 (5'-AGCCTCATCCCCAACCTGAGGCACG-3 normal allele) or 9.4 (5'-AGCCTCATCCCCAACCTGAGGCACA, mutant allele). The underlined base was deliberately destabilized (it should have been A) in order to ensure allele-specificity. Three to five μl of rapid prep DNA (Talmud et al. 1991, see 2A.2.2) was added to 50 pmol of each of the appropriate primers, 100 μmol of each deoxynucleoside triphosphate, 5 μl of 10x Mg^{2+}-free Taq DNA polymerase buffer (Promega) supplemented with 15 mmol $MgCl_2$ and 2 units sequencing grade Taq (Promega) in a final volume of 50 μl. Samples were overlaid with light mineral oil(Sigma) and cycled 30 times (93°C for 1 min, 70°C for 1 min, 72°C for 2 min). Half of each PCR product was analysed on 6% acrylamide (See Figure 2A.5).

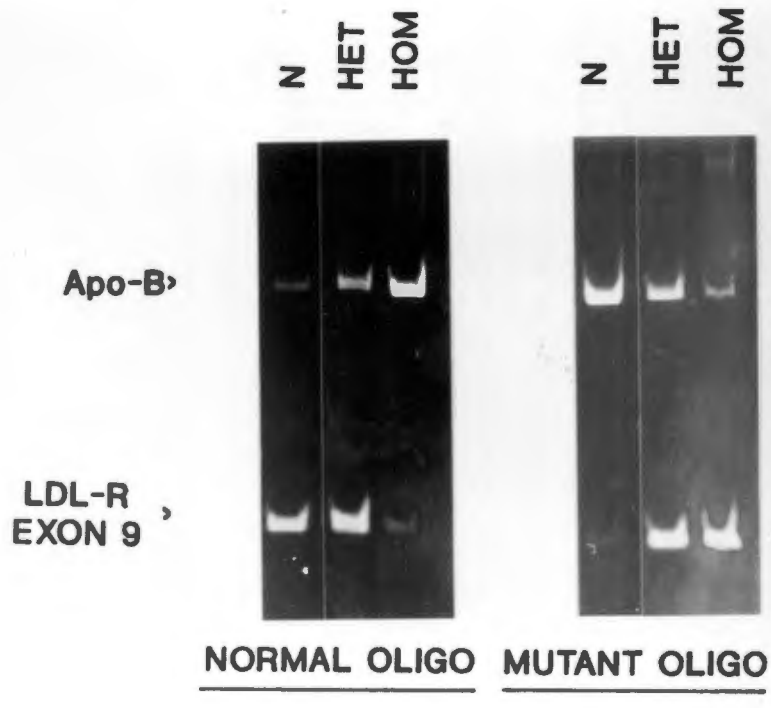


Fig. 2A.5: ARMS assay for the detection of the FH-Afrikaner-3 mutation. Genomic DNA was amplified with oligonucleotides AB-1 and AB-2 (internal controls for PCR reaction that amplify the Apo B gene) and 9.5 with either 9.3 (that hybridises to the normal LDL-receptor) or 9.4 (that hybridises to this mutation in exon 9 of the LDL-receptor. PCR products were electrophoresed on 6% Acrylamide). N = normal, HET = heterozygote for FH-Afrikaner-3 mutation, HOM = homozygote for FH-Afrikaner-3 mutation.

The advantage of this assay is that it provides amplified apo B-100 product that can be analysed for the apo B-3500 mutation (FDB) by allele-specific oligonculeotide hybridisation exactly as described by Tybjaerg-Hansen et al. (1990). This assay was used to screen the Afrikaner and Coloured patients for FDB.

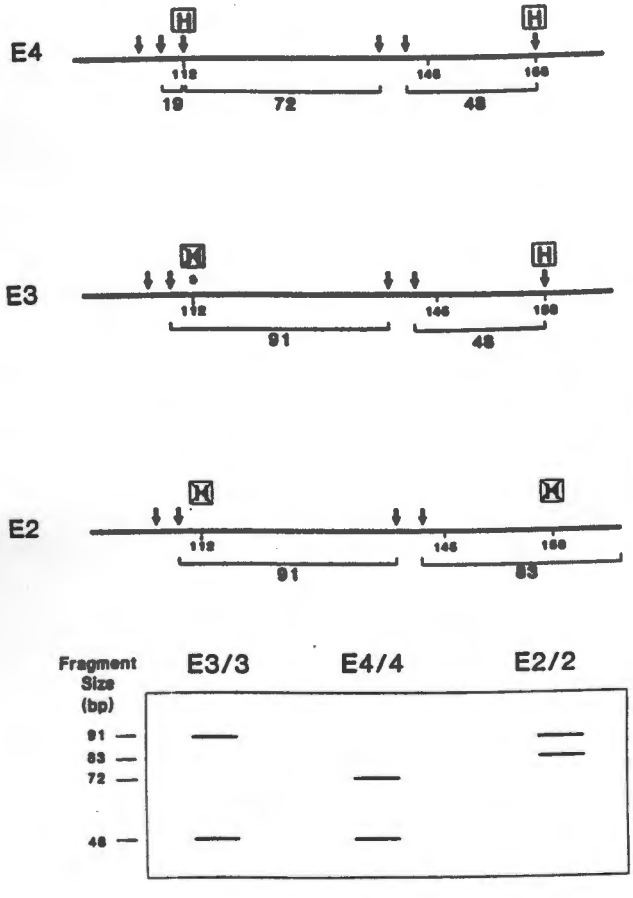
2A.9.8 Restriction isotyping of apolipoprotein E polymorphisms

This assay was carried out as described by Hixson and Vernier(1990). A region of the apo E gene flanking the polymorphic codons 112 and 158 was amplified with oligonucleotides G23 and G24 (see Table 2A.1). Forty microlitres of the PCR product were digested overnight at 37°C with Hha I (6u, Promega) without the addition of any buffers and was electrophoresed on 10% polyacrylamide gels. Hha I digests the 244 b.p. PCR product at 3 invariant sites in the E2 allele but cleaves in addition at codon 158 in the E3 allele, and at codons 112 and 158 in the E4 allele (Fig. 2A.6).

2A.10 CLONING

2A.10.1 Preparation of Competent cells

JM 85 cells were kindly provided by Prof. D. Botes (Department of Biochemistry, UCT). 40 ml of LB were innoculated with 2 ml of an overnight culture of JM 85 cells. These cells were grown for 2 hours till they reached an OD_{550} of 0.25. The bacteria were pelleted by centrifugation at



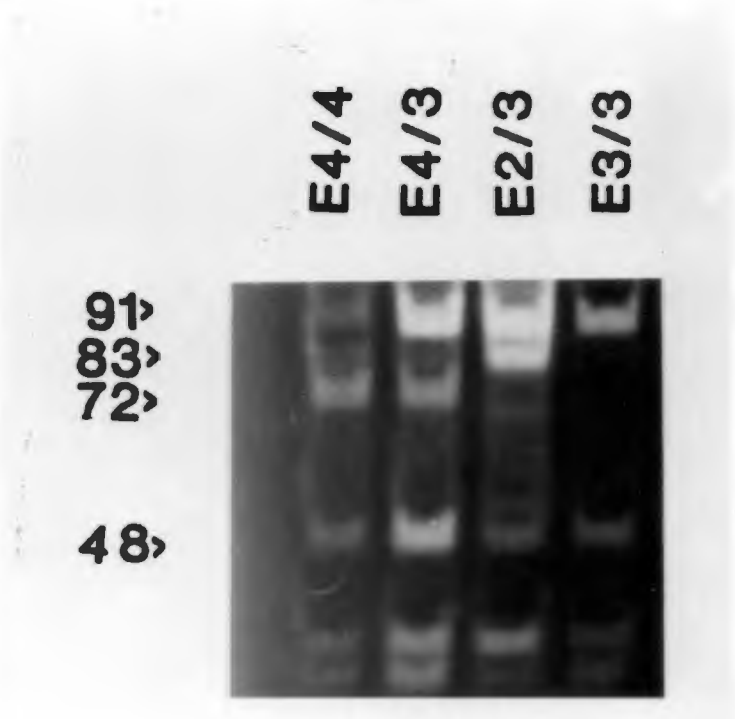


Fig. 2A.6: Assay for Apo E genotyping with Hha I. Genomic DNA was amplified by PCR using oligonucleotides G23 and G24 that span the polymorphic codons 112 and 158 of the apo E gene (see table 2A.1). PCR products were digested overnight at 37°C with Hha I (6 u) and were electrophoresed on 10% polyacrylamide gels. The enzyme Hha I differentiates the polymorphisms at codons 112 and 158.

3000 rpm at 4°C in a Beckman JA-20 rotor. This pellet was resuspended in 20 ml ice-cold solution of 60 mM $CaCl_2$, 10 mM Pipes, pH 7.2, left on ice for 30 min then re-pelleted by centrifugation at 3000 rpm at 4°C for 5 min. This pellet was resuspended in 4 ml 60 mM $CaCl_2$, 10 mM Pipes pH 7.2 and left on ice for at least 30 minutes prior to using for transformation.

2A.10.2 Restriction digestion and ligation of PCR product and vector

Exons 1 to 4 of the LDL-receptor cDNA, prepared as described earlier (2A.4), were amplified using AR12 and AR13 (see Table 2A.1, Fig. 4.8). The PCR products were electrophoresed on a 6% acrylamide gel and the appropriate bands were cut out of the gel and eluted as described earlier (2A6.1). This fragment and pUC 118 were both simultaneously digested with Bam HI (11 u) (Boehringer Mannheim, Mannheim, Germany) and Sph I (12 u) (Boehringer Mannheim) in the Boehringer Mannheim Buffer B. The digested pUC 118 was separated from the released insert on 1% agarose and the vector was eluted from the gel with Geneclean II (Bio 101, La Jolla, CA, USA).

The digested products were ligated overnight at 16°C using 3u T4 DNA ligase (Promega Madison, WI, USA) in the supplied T4 ligase buffer.

10 μl of the ligated product was mixed with 40 μl of a 10 mM Tris-HCl, pH 8.0, 25 mM $CaCl_2$ solution and this mix was added

to 100 µl ice-cold competent cells. These cells were then heat-shocked at 42°C for three minutes and left to cool at room temperature for 10 min. After adding 850 µl LB (Sambrook et al. 1989), this mix was placed at 37°C in a rotary shaker for 45 min. 40 µl of X-gal (20 mg/µl in dimethylformamide) was spread on premade LB plates (Sambrook et al. 1989) containing Ampicillin (50 µg/ml) and placed at 37°C for about 1 hour prior to plating (JM85 cells only need the addition of X-gal for α-complementation).

200 µl aliquots of the cells were spread onto LB plates containing Ampicillin and incubated at 37°C overnight.

White colonies were picked and minipreparations of DNA for sequencing were obtained by alkali-lysis exactly as described (Sambrook 1989, pp 1.25-1.28). DNA from colonies was tested for the presence of insert by digesting about 20% of each minipreparation with Eco R1 (14 µ) (Promega Madison, WI) and Hind III (11 µ Boehringer Mannheim) in Buffer B (Boehringer Mannheim) to see if the appropriate size insert was released.

2A.11 Northern Blots

Northern transfer and hybridization was performed essentially as described (Selden 1989). The following minor alterations were introduced. 40 ug of each sample of total RNA was electrophoresed on 1% Agarose gels containing 1 x MOPS and 1.1% formaldehyde. RNA was blotted as described onto HYBOND™-N (Amersham, Buckinghamshire, UK). The filter was

baked at 80°C for 2 hr. The blot was prehybridised at 42°C for 2 hr and hybridised overnight at 42°C in the described solutions with pLDLR-2HH1 (Taylor et al. 1988) that had been random primed (Multiprime DNA labelling system, Amersham). Membranes were exposed to X-ray film to visualise the bands.

2A.12 Oligonucleotide purification

Some of our oligonucleotides that were size-degenerate were purified with the following method for use in sequencing.

Oligonucleotides were dissolved in 200 µl sterile d H_2O and dried down in a Speedvac (Savant Instruments Inc). The dried oligonucleotides were resuspended in 500 µl H_2O and dried. This was repeated again. (This process removes the ammonium as the oligonucleotides are initially prepared in concentrated ammonium hydroxide). The pellet was redissolved in 400 µl deionized formamide. 300 µl of this solution was stored for future use at -20°C and 100 µl was electrophoresed on a 20% acrylamide 7% urea preparative gel. The gel was pre-electrophoresed for 30 minutes at 200 V then the samples were loaded and electrophoresed at 300 V for 4 hours. The oligonucleotides were localized on the gel by UV shadowing on TLC paper. The band was cut out from the gel with a new steel blade and crushed through a 5 ml syringe into a 10 ml sterile tube. The oligonucleotides were extracted from the gel by incubating it with 10 ml 0.3 M sodium acetate pH 5.6, 1 µM EDTA at 37°C overnight. The slush was then centrifuged at 2000 rpm for 10 min. The liquid was carefully poured off

and the oligonucleotides were desalted and concentrated using C18 Sep-pack columns (Waters Associates, Mifford Massachusetts USA). These columns were charged by washing with 10 ml HPLC grade acetonitrile (Rathburn Chemicals Limited, Walkerburn, Scotland) followed by 10 ml sterile H_2O. The aqueous solutions containing the oligonucleotides were loaded onto the column and the column was then washed with 10 ml H_2O. The oligonucleotides were eluted with 60% methanol; 4 ml 60% methanol was passed through the column and 1 ml aliquots were collected. The first 2 aliquots were dried and resuspended in water. After confirming that most of the oligonucleotide was in the first aliquot by measuring the OD_{260}, the oligonucleotides were freeze dried and reconstituted in H_2O in 20 μM stocks.

2A.13 Lipoprotein preparation and iodination

Human LDL (Goldstein et al. 1983), Rabbit ß-VLDL (Kovanen et al. 1981), IgG C7 (Beisigel et al. 1981) and human lipoprotein deficient serum (Goldstein 1983) were prepared as described. Iodination of LDL, ß-VLDL (Goldstein et al. 1983) and IgG-C7 (Beisigel et al. 1981) with ^{125}I was performed as described. The specific activity of LDL, IgG-C7 and ß-VLDL ranged from 214-748 cpm/ng, 480-1442 cpm/ng and 618-1581 cpm/ng respectively. Detailed descriptions of the above can be found in the thesis of A.M. Fourie (1989).

2A.14 Cell Culture

Skin fibroblasts were usually seeded at 50 000 or 60 000 cells per 60 mm Petri dish (ligand binding experiments and some immunoprecipitation experiments) or at 20 000 cells per 35 mm diameter Petri dish (some immunoprecipitation experiments) in Dulbecco's MEM (DMEM) (Flow Laboratories, Ayrshire, Scotland, UK) containing 10% (v/v) fetal calf serum (day 0). On day 3 this medium was replenished. On day 5, the medium was changed to Dulbecco's MEM containing lipoprotein-deficient serum (LPDS) (5 mg protein/ml). This medium was replenished on day 6. Experiments were performed on day 7. Occasionally the number of cells seeded per dish or the number of days of cell growth in DMEM containing fetal calf serum were varied in order that cells were subconfluent and thus still rapidly dividing at the time that they were placed in lipoprotein-deficient medium so that maximal upregulation of LDL receptors should occur.

2A.15 LDL binding and metabolism in fibroblasts as assessed with DiI-LDL

The fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was incorporated into LDL (Pitas 1981). One mg of LDL in 2 ml of LPDS was vortexed for 10-15 sec with 50 μl of a 3 mg/ml DiI solution in dimethylsulphoxide. DiI was incorporated into the lipid phase for 15 hr at 37°C. The density of the incubation mixture was raised to 1.063 g/ml and DiI-labelled-lipoproteins were isolated by ultracentrifugation at 40 000

rpm for 24 hr (Beckman SW40Ti Rotor). The LDL in the top 6 mm was collected and dialyzed against 0.9% NaCl, 0.01% EDTA for 48 hr. The DiI-LDL was sterilised by filtration through a 0.22 μM filter (Millex GU, Millipore) prior to storage at 4°C. The protein concentration of the DiI-LDL was determined (Lowry et al. 1951).

DiI-LDL (20 μg/ml) was incubated with upregulated cells for 4 hours at 37°C and visualised by fluorescence microscopy (Sanan 1985).

2A.16 Surface binding of ^{125}I-ß-VLDL to fibroblasts at 4°C

Surface binding of ^{125}I-ß-VLDL at 4°C was performed at 1 μg/ml ^{125}I-ß-VLDL with or without the simultaneous addition of 100 μg/ml unlabelled ß-VLDL. Fibroblast monolayers were washed once with Dulbecco's phosphate buffered saline (Ca^{2+} and Mg^{2+}-free) (PBS), then the ß-VLDL was added to the cells in MEM, buffered at pH 7.4 with 20 mM HEPES, containing LPDS (5mg protein/ml) for 2 hr at 4°C. After the medium was collected the cells were washed 4 times with PBS containing 0.2% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany) followed by 3 washes with PBS at 4°C. Six hundred μl PBS was added to each 60 mm dish and the cells were scraped off the dishes with cell scrapers and collected into 1.5 ml microfuge tubes. The cells were centrifuged at 12 000 g for 5 minutes in a microfuge. The ^{125}I-ß-VLDL bound to the cells was considered to be associated with the pelleted cells

that were counted after the supernatant was carefully removed.

2A.17 Surface Binding of ^{125}I-IgG-C7 or ^{125}I-LDL to fibroblasts at 4°C

Surface binding of ^{125}I-IgG-C7 (Fourie et al. 1988) and ^{125}I-LDL (Grant et al. 1989) was performed at 4°C using the indicated concentrations of the respective ligands. Prior to adding ligands to the dishes the cells were washed once with Dulbecco's Phosphate buffered saline (PBS) (Ca^{2+} and Mg^{2+}-free). Incubations were done in MEM, buffered at pH 7.4 with 20 mM HEPES, containing LPDS (5mg protein/ml) . After 2 hours incubation the cells were washed 4 times with PBS containing 0.2% bovine serum albumin followed by 3 washes with PBS at 4°C. In the ^{125}I-LDL binding experiments 2 ml of heparin (4 mg/ml) were added to the cells for 40 minutes at 4°C and the ^{125}I-LDL released was taken as the bound fraction. In the ^{125}I-IgG-C7 experiments, the cell-associated radioactivity was quantitated after the cells were dissolved in 1 N NaOH.

2A.18 ß-VLDL affinities at 4°C

The affinities of the LDL receptors for Apo E were estimated by measuring the ability of unlabelled ß-VLDL to compete for ^{125}I-LDL binding at 4°C. Rabbit ß-VLDL was added at the indicated concentrations to 2 µg/ml ^{125}I-LDL in MEM, buffered at pH 7.4 with 20 mM HEPES, containing LPDS (5mg protein/ml). These premixes were added to dishes and ^{125}I-LDL binding was

determined as above. The ß-VLDL concentration required for half-maximal inhibition of ^{125}I-LDL binding was taken to reflect the affinity of the receptors for ß-VLDL. This value was determined by non-linear regression analysis (Enzfitter; Elsevier Biosoft, Cambridge, U.K.).

2A.19 ^{125}I-LDL and ^{125}I-ß-VLDL metabolism in fibroblasts at 37°C

The ability of cells to bind, internalize and degrade ^{125}I-LDL and ^{125}I-ß-VLDL at 37°C was measured at the indicated ligand concentrations as described previously (Goldstein et al. 1983). Prior to adding ligand to the dishes, the cells were washed once with PBS. After the cells were incubated with the desired concentration of ^{125}I-LDL or ^{125}I-ß-VLDL in DMEM/LPDS at 37°C for 4 hours, they were put on a cold metal tray in the 4°C cold room. The medium was immediately harvested so that its content of non-iodide trichloroacetic acid-soluble radioactivity could be measured for LDL or ß-VLDL degradation. The cells were washed four times with PBS-albumin and three times with PBS. The cells that had been incubated with LDL were then treated with heparin as described in the 4°C LDL binding assay. The cells that had been incubated with ß-VLDL were treated with 10 mM suramin (Germanin, Bayer-Miles, South Africa), 50 mM NaCl, 3 mM $CaCl_2$, 10 mM Hepes pH 7.4 for 1 hour at 4°C. The heparin- or suramin-releasable radioactivity was taken as the bound fraction. The cells were then solubilized in 1 M NaOH and the radioactivity quantitated (intracellular ^{125}I-LDL or

^{125}I-β-VLDL). Cellular protein in the solubilized extract was also determined (Lowry et al. 1951). Degraded ^{125}I-LDL or ^{125}I-β-VLDL in the medium was determined in the following way: The protein was precipitated from the medium by the addition of 0.3 volumes 50% (w/v) trichloroacetic acid and by incubation at 4°C for 30 minutes. The precipitate was sedimented by centrifugation at 2000 rpm (Beckman TJ-6 centrifuge) for 30 minutes at 4°C. An aliquot of the supernatant (1 ml) was mixed with 20 µl 40% potassium iodide and 50 µl 30% hydrogen peroxide that converts free iodide to iodine. The free iodine was extracted with 2 volumes of chloroform and the radioactivity in the aqueous phase was considered to represent the degraded lipoprotein ligand.

2A.20 Calculation of B_{max} and K_d values

The B_{max} and K_d values were calculated from binding curves using a commercial software package (Enzfitter; Elsevier Biosoft, Cambridge UK) for non-linear regression analysis assuming one saturable site plus non-saturable processes. In some cases specific values were determined by subtracting from the total activity the non-specific values obtained either in the presence of excess unlabelled LDL (200 µg/ml) or in cells in which LDL receptor activity had been fully downregulated by treatment with 1 µg/ml 25-OH cholesterol for 48 h. The resultant curves of specific high-affinity activity were analyzed using Enzfitter assuming only one site of specific binding.

2A.21 [^{35}S]-Methionine pulse-chase experiments

These experiments were done essentially as described (Tolleshaug et al. 1982). On day 7, upregulated cells were pre-incubated for 30 minutes in methionine-free Eagles MEM containing LPDS (5mg protein/ml) at 37°C, after which cells were pulse-labelled at 37°C for the required time with [^{35}S]-methionine (50 - 100 μCi/ml; Methionine, L-[^{35}S]-, Dupont, Wilmington, DE, USA). The medium was then changed to DMEM/LPDS containing 200 μM unlabelled methionine and the cells were chased for varying times. The cells were then washed and solubilized. Post-nuclear supernatants were incubated for 1 hour at 4°C with a preformed immune complex containing IgG-C7 (mouse monoclonal anti-LDL receptor antibody) coupled to a goat anti-mouse antibody (Organon Teknica Corp. West Chester, PA, USA). The immunoprecipitates were pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded or kept aside for subsequent transferrin receptor immunoprecipitation. The pellets containing the immunoprecipitates were washed through a 4-step sucrose density gradient where each layer contained a different combination of detergent and ionic strength. Immunoprecipitates were electrophoresed on 7% SDS polyacrylamide gels which were enhanced with salicylate, dried and exposed to X-ray film (Kodak X-OMAT-AR). The ^{35}S radioactivity in the mature and precursor LDL receptors was quantified by densitometric scanning of the fluorograms (Cliniscan, Helena Laboratories, Beaumont, Texas, USA).

In some experiments, transferrin receptors were immunoprecipitated in addition to LDL receptors to serve as a control for protein synthesis in different cell types. The transferrin receptors were immunoprecipitated from the supernatant left after LDL-receptor immunoprecipitation with a mouse anti-transferrin antibody-goat anti-mouse antibody complex in exactly the same way as the LDL receptors.

2A.22 Determination of [^{35}S]-methionine incorporation into total trichloroacetic acid-precipitable protein

In some experiments we needed to determine [^{35}S]-radioactivity incorporated into total trichloroacetic acid-precipitable cell protein during a pulse-labelling period as a measure of total protein synthesis. We used the filter disc method outlined briefly below (Mans and Novelli 1961, Bester 1973). An aliquot of the [^{35}S]-labelled post-nuclear supernatant from the cell-lysate obtained as described in 2A.21 (Tolleshaug et al. 1982) was diluted in 9 volumes H_2O and then applied to a Whatman GF/C filter. This filter was successively immersed in 10% trichloroacetic acid (TCA) at 4°C for 30 minutes, 5% TCA for 15 minutes at room temperature, 5% TCA at 90°C for 20 minutes, 5% TCA at room temperature for 5 minutes, ethanol : diethyl ether (1:1) for 5 minutes, and diethyl ether for 5 minutes. The ^{35}S-labelled TCA-precipitable protein on the filter disc was then measured by scintillation counting (Beckman Ready-Solv™ EP scintillation cocktail, Packard 4690 Scintillation counter).

SECTION B: AN EXPLORATION OF THE SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP) METHOD FOR THE DETECTION OF POINT MUTATIONS.

2B.1.1 Introduction: Techniques other than SSCP that are used to detect point mutations .

The ability to detect point mutations has become very important in genetic research. Techniques that can screen large stretches of DNA or RNA for mutations without having to resort to sequencing have the potential to easily detect new mutations, to screen populations for new and previously detected mutations, and can be used to detect new markers for gene mapping independent of the sequence constraints imposed by restriction endonuclease sites.

Many different approaches have been developed to solve this problem. Some methods have relied on the susceptibility to chemical modification and/or enzyme digestion of exposed unpaired bases, formed by annealing a mutant to a normal sequence . Earlier techniques included attempts to cleave DNA:DNA mismatches with the single-strand specific nuclease S1 (Shenk et al. 1975, Dodgson and Wells 1977) or by the cleavages of RNA:DNA mismatches by ribonuclease A (Myers et al. 1985). The problem with these methods was that they only detected about 50% of mutations (Myers et al. 1985). Recent variations of this approach have relied on the abilities of hydroxylamine and osmium tetroxide to modify mismatched C's and T's, respectively, and make them susceptible to piperidene cleavage (Cotton et al. 1988). Theoretically,

this technique could be virtually 100% sensitive to point mutations (Rossiter and Caskey 1990). However, the chemical method has been unable to detect some mutations that have been detected by other methods (Theophilus et al. 1989).

Another group of techniques involve denaturing gradient gel electrophoresis. This method relies on the principle that different DNA sequences, even those that differ by only one base pair, have different melting temperatures. Normal and mutant duplex fragments migrate through acrylamide gels containing a gradient of formamide, urea, or temperature with a constant mobility as a function of their molecular weight, while they are double stranded. When they reach the region of the gel that has sufficient denaturant to cause melting, the rate of migration is suddenly slowed. Theoretically, at least, the differences in melting temperature between two sequences differing by a single base pair or a native double-stranded sequence and a heteroduplex with a single base-pair mismatch, should allow resolution of single base-pair changes on denaturing gradient gels (Rossiter and Caskey 1990). A problem with this method was that it did not pick up mutations in regions of the sequence that melted latest, because at that stage the strands had already started to separate and the electrophoretic mobility had been arrested to some extent. To circumvent this and improve the sensitivity of the technique a GC-rich region was placed at one end of the sequence to be analysed in order that this so-

called GC clamp should be the last area of the sequence to denature.

The immunomicroscopy technique (Ganguly et al. 1989) relies on the binding of carbodiimide to the point of mismatch in a heteroduplex. The carbodiimide is then detected with a polyclonal anti-carbodiimide antibody which is itself recognised by a second antibody linked to an electron dense marker. This complex is ultimately visualised by electron microscopy. The advantage of this technique is that it can identify mutations in large lengths of DNA (e.g. 7 kB) but can only locate mutations with an accuracy of 0.1 kB to 0.3 kB. This technique seems to be quite finnicky and time-consuming as there are difficulties in detecting A:C/G:T or G:G/C:C mismatches. In addition, false positives from non-specific labelling of molecules have to be ruled out (Ganguly et al. 1989).

The altered electrophoretic mobility of heteroduplexes as opposed to homoduplexes can often be detected on Hydrolink gels (AT Biochem. Inc., Malvern, PA, USA) (Keen et al. 1991). This is another way to screen PCR products for point mutations or small deletions (Keen et al. 1991). Heteroduplexes form in heterozygous samples as part of the PCR reaction (as soon as the efficiency of amplification starts to plateau) and can be artificially created from two different homozygote PCR products by mixing, denaturation and renaturation. A modification of this method was used to

detect the 3 b.p. deletion in exon 4 of the LDL receptor commonly found in Ashkenazi Jews (Meiner 1991). (See Section 2A.9.5.)

2B.1.2 Introduction to the Single-Stranded Conformational Polymorphism (SSCP) method for detecting point mutations.

Recently, a method has been developed that relies on the observation that the electrophoretic mobility on non-denaturing gels of single-stranded DNA is determined by its conformation that is in turn a function of both the size and sequence of the molecule. This technique uses PCR spiked with [α^{32}P]-dCTP or a kinased oligonucleotide to generate DNA sequences that are then denatured in a formamide buffer prior to electrophoresis in non-denaturing gels under a variety of conditions (Orita et al. 1989a, Orita et al. 1989b). The temperature of electrophoresis, the concentration of TBE in the running buffer and in the gel, and the glycerol concentration in the gel can all be varied to change and optimise the banding patterns resulting from the single stranded DNA.

SSCP is one of the easiest and quickest methods available to detect point mutations, but not much work has been done to ascertain its sensitivity. This technique was investigated as it would be potentially of use in the initial detection of point mutations in the Indian FH homozygotes and subsequent population screening.

The apo E polymorphisms were used to test the sensitivity of point mutation detection of SSCP gels. The apo E gene has 3 common alleles. The commonest allele E3 allele has a cysteine at codon 112 and an arginine at codon 158, the E4 allele has arginines at both codons and the E2 allele has cysteines at both sites. We had been using the Hha 1 restriction digestion system (Hixson and Vernier 1990) to distinguish genotypes and combinations of the apo E2, E3, E4 alleles from each other (see Section 2A.9.8, and Fig. 2A.6). HhaI recognises the sequence GCGC that is present when either codon 112 or 158 are arginines (GCG) but not when they are cysteines (TGC). Unfortunately this method was designed to detect polymorphisms at codons 112 and 158 in the apo E gene, and does not detect mutations at many other sites. In addition to the potential of developing a system for rapid apo E genotyping, we also wanted to develop a system to distinguish the Arg_{145}-Cys and Lys_{146}-Gln mutations that had been identified in our laboratory (W.J.S. de Villiers, G.A. Coetzee, D.R. van der Westhuyzen, personal communication) from the common apo E alleles E2, E3 and E4 as this could not be accomplished by Hha I restriction isotyping.

Single-stranded versions of the different apo E alleles at our disposal and combinations thereof were electrophoresed on various types of SSCP gels in order to see which gel(s) resolved the polymorphisms best and whether it would be feasible to use SSCP as an apo E genotyping system and as a means of identifying mutations in our Indian FH patients.

2B.2 Methods

A 244 b.p. fragment of the apo E gene that encompassed the polymorphic sites at codons 112 and 158 was amplified using oligonucleotides G23 and G24 (see Table 2A.1). The PCR was labelled by adding 1-10 μCi (0.1 - 1 μl) [α-^{32}P]-dCTP (Amersham, UK) to the mix. On certain occasions the PCR product was labelled by kinasing one of the oligonucleotides in the mix with [γ-^{32}P]-ATP. One to three μl of the PCR product was mixed with 9 μl of 95% deionised formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. This was heated at 95°C for 3-5 minutes, placed immediately on ice and then loaded onto the gel.

Gels were run on a conventional sequencing apparatus (40 mm x 20 mm x 0.4 mm) with a cooling plate. The gels were cooled with a fan when run at room temperature. Gels were composed of 6 or 8% acrylamide, 0.5x or 1xTBE, and 0%, 5% or 10% Glycerol. These gels were made as 50 ml solutions. Five ml was taken to form a plug and was polymerised with 500 μl 3% ammonium persulphate and 5 μl TEMED. After the plug had set, the remaining 45 μl was polymerised with 2.5 ml 3% ammonium persulphate and 25 μl TEMED. Gels were pre-electrophoresed for an hour at the desired temperature (4°C in the cold room, or room temperature) and power prior to loading samples. Gels were run at constant power as shown in Table 2B.1. The samples were normally electrophoresed at least until the bromophenol blue reached the bottom of the gel.

Table 2B.1: Comparison of different conditions for SSCP analysis of apo E2, E3, E4 and Arg_{145}-Cys alleles in homozygote and heterozygote combinations.

% Acrylamide	6	6	6	8	6	8	8	6	6	6	8	50% Hydrolink
TBE	0.5	1	1	1	1	1	0.5	0.5	0.5	0.5	0.5	0.6
% Glycerol	5	10	0	0	10	10	5	10	0	5	0	0
Temp	Room	Room	Room	4°C	4°C	Room	Room	Room	4°C	4°C	4°C	Room
Watts	40	40	8	8	8	8	40	40	8	8	8	8
Alleles Compared												
E2/E3	++	+	+	-	+	+	++	+	+	+	+	+
E3/E4	+	++	-	-	-	-	-	-	+	+	++	-
E2/E4	+	++	+	-	+	+	++	+	-	-	+	+
145/2	++	+	-	+	+	-	+	-	++	++	++	++
145/3	++	+	+	+	+	+	+	-	+	+	+	++
145/4	+	++	+	+	+	+	+	-	++	++	++	-

NOTE: All PCR products labelled with [α^{32}]-dCTP

Legend:
- = no clear difference in banding pattern of homozygotes for the two alleles being compared.
+ = homozygotes for the two alleles have different banding patterns but the heterozygote of the combination of the two alleles cannot be differentiated from one or both of the homozygote patterns.
++ = homozygotes for the two alleles and the heterozygote combination of the two alleles are all clearly distinguishable from each other.

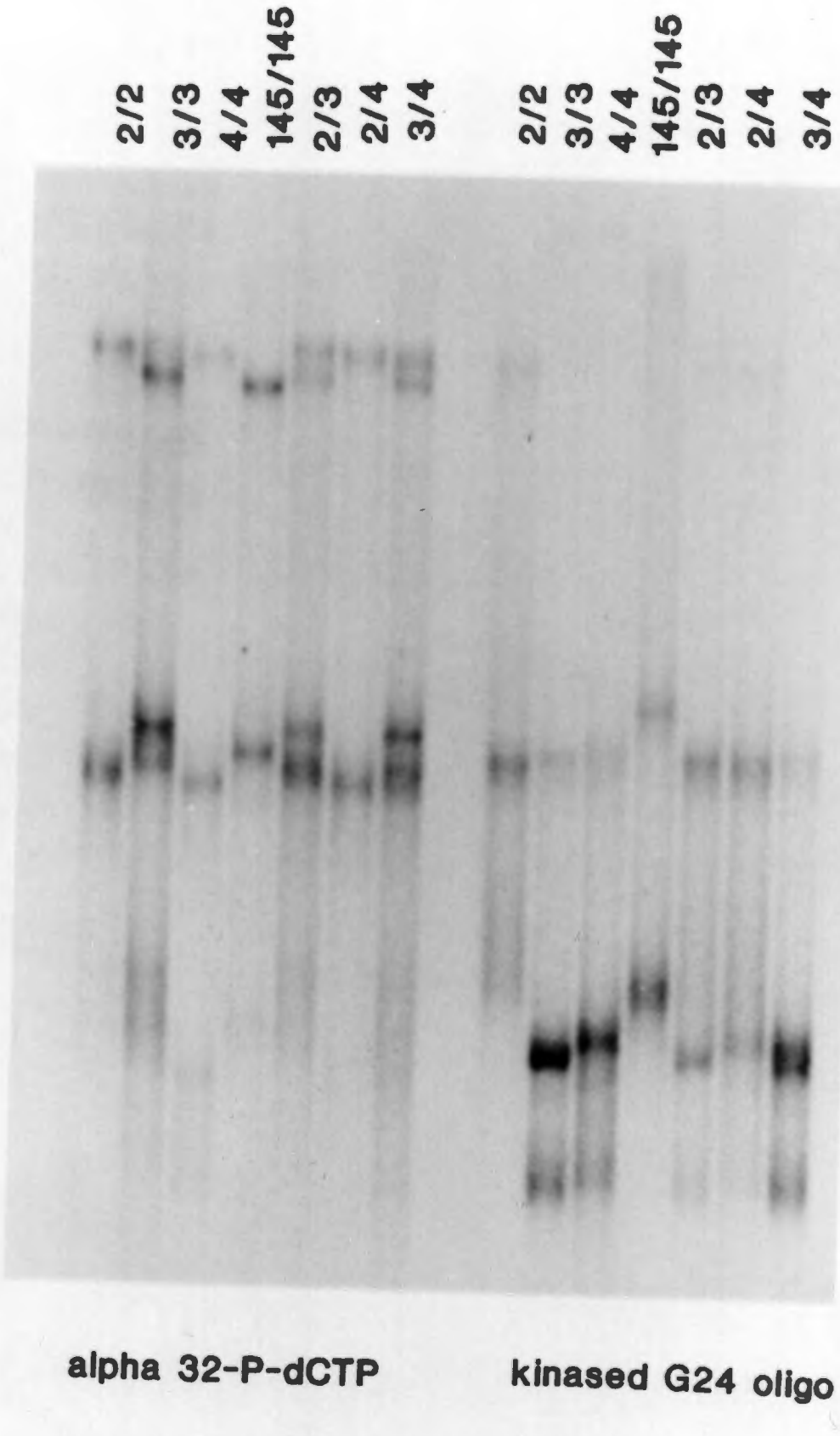


Fig. 2B.1: Comparison of SSCP patterns of PCR products of apo E genotypes labelled either with [α-^{32}P]-dCTP or with a kinased primer. PCR of the apo E gene was performed as described using G23 and G24 (see table 2A.1). PCR products were radioactively labelled either by the addition of [α-^{32}P]-dCTP or some kinased oligonucleotide G24 to the PCR mix. PCR products were denatured in formamide and were electrophoresed at 4°C at 8 W on a SSCP gel containing 8% acrylamide, 0% glycerol and 0.5x TBE. 145 = Arg_{145}-Cys apo E mutation.

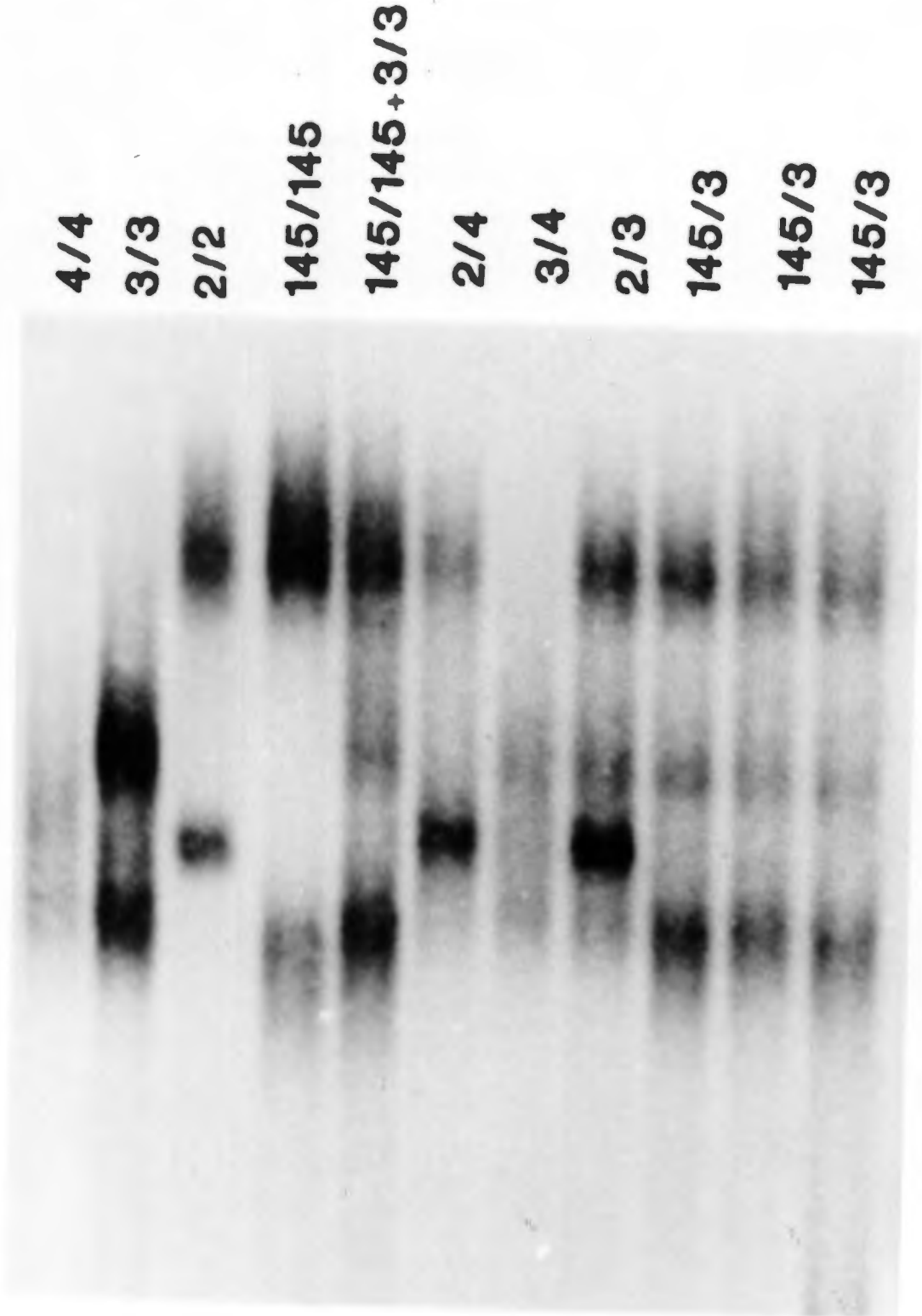


Fig. 2B.2: Diagnosis of the Apo E Arg_{145}-Cys mutation on a SSCP gel. PCR of the apo E gene was performed as described using oligonucleotides G23 and G24 (see table 2A.1). The PCR products were radioactively labelled by the addition of [α-^{32}P]-dCTP to the PCR mix. PCR products were denatured in formamide and were electrophoresed at 40 W at room temperature on a 6% gel containing 5% glycerol and 0.5 x TBE. The lane labelled 145/145 + 3/3 is a mix of PCR products from a 145/145 and a 3/3 homozygote. The proband was a 145/145 and his three children were 145/3. 145 = Arg_{145}-Cys mutation.

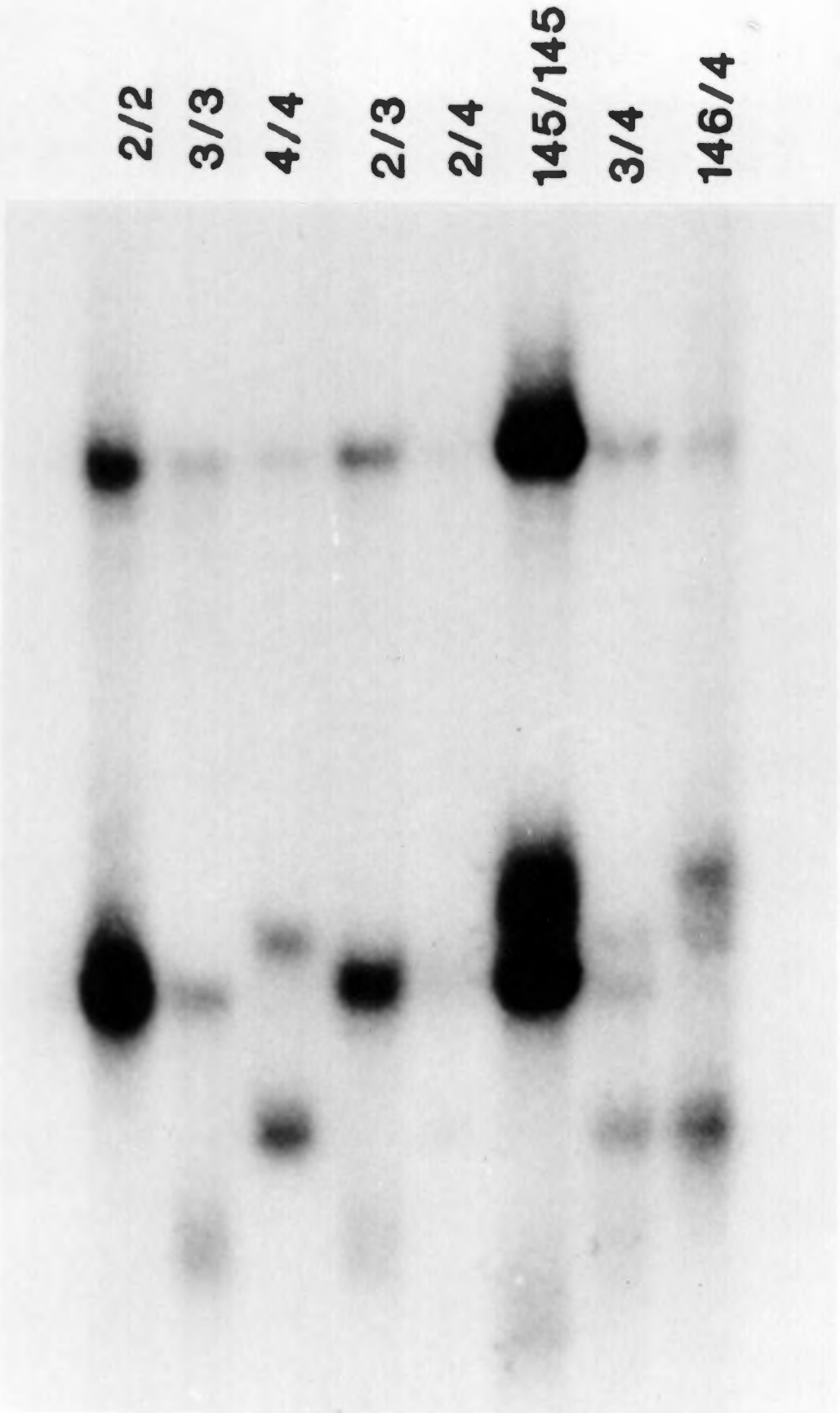


Fig. 2B.3: Diagnosis of the Apo E Lys_{146}-Gln mutation on SSCP gels. Radiolabelled PCR products of the above apo E genotypes were generated by PCRing genomic DNA with oligonucleotides G23 and G24 (see table 2A.1) and [α-^{32}P]-dCTP. PCR products were denatured in formamide and were electrophoresed at 4 W at room temperature on a 6% acrylamide gel containing 1 x TBE and 10% glycerol. 145 = Arg_{145}-Cys; 146 = Lys_{146}-Gln.

In some cases, hydrolink gels (MDEtm, AT Biochem Inc, Malvern PA, USA) were used instead of conventional acrylamide. The hydrolink gel was made in a final volume of 60 ml consisting of 15 ml Hydrolink, 41 ml H_2O, 3.6 ml 10x TBE and was polymerised with 0.3 ml ammonium persulphate and 0.03 ml TEMED.

Gels were dried at 80°C for 1-2 hours and then were exposed to X-ray film (Kodak X-OMAT-AR).

2B.3 Results and Discussion

Table 2B.1 summarises our experience in distinguishing the apo E2, E3, E4 alleles and the Arg_{145} to Cys mutation from each other. The most successful gels are shown in figs. 2B.1, 2B.2 and 2B.3. It was clear from this series of gels that the sharpness/smudginess of the banding patterns and the ability to differentiate between different alleles varied from gel to gel (see Table 2B.1 and Figs. 2B.1, 2B.2, 2B.3).

SSCP patterns obtained from PCRs of a given genotype labelled with [α^{32}P]-dCTP were compared to those obtained when the PCR was labelled by the incorporation of a kinased primer (Fig. 2B.1). The α-labelling incorporates radioactivity into both strands synthesized in the PCR reaction; in the ideal situation 2 bands should be visible in a homozygote subject and 4 bands from a heterozygote. The PCR reaction labelled with a kinased oligonucleotide primer only incorporates radioactivity into the strand primed by that oligonucleotide.

Consequently, in this scenario one expects one band from a homozygote and 2 bands from a heterozygote. Unfortunately, the number of bands seen is confounded by 2 situations. Firstly, a given single strand can migrate at more than one mobility, presumably due to folding into more than one stable conformation (Orita 1989a). Secondly, 2 complementary strands can run with the same electrophoretic mobility.

None of the SSCP gels tested allowed absolutely successful apo E isotyping when the PCR products were labelled with [α^{32}P]-dCTP (Table 2B.1). Many of the gels could successfully differentiate the homozygotes from each other. However, the distinction of heterozygotes from the homozygotes that had one of the alleles of the heterozygote was often impossible. In Fig. 2B.1, for example, it was impossible to distinguish a 2/4 from a 4/4 in the [α^{32}P]-dCTP labelled samples. The results were more promising when the PCR was labelled with kinased G24. This approach differentiated between most of the apo E variants in both homozygous and heterozygous forms (Fig. 2B.1).

An interesting feature emerged when the same DNA, PCRed either with [α^{32}P]-dCTP, or with a kinased oligonucleotide, was electrophoresed on the same gel (Fig. 2B.1). The mobilities of the PCR products labelled with a kinased oligonucleotide were not the same as those of the corresponding [α^{32}P]-dCTP-labelled samples. The addition of a 5' phosphate and the resulting extra negative charges

probably result in different single stranded conformations and/or the extra charge contributes to an increased electrophoretic mobility.

One of the useful applications of this work on SSCP of apo E was the development of assays that differentiated the Arg_{145}-Cys and Lys_{146}-Gln mutations from the wild type alleles E2, E3 and E4. Hha I analysis of the Arg_{145}-Cys mutation (when it occurs on an E3 allele) is undistinguishable from E3, since this mutation does not create or destroy a Hha 1 restriction site. A patient homozygous for the Arg_{145}-Cys mutation, originally described by Rall et al. (1982), was detected in our laboratory (W.J.S. de Villiers, personal communication). It was important for us to know whether the 3 children of this patient had the mutation or not in order to establish the dominant or recessive nature of this mutation. All three children's DNA typed as E3/E3 when analysed by Hha I digestion. The SSCP analysis of the 3 children (Fig. 2B.2) showed clearly that these 3 children were E3/Arg_{145}-Cys heterozygotes.

A similar situation arose with a patient who was heterozygous for the Lys_{146}-Gln mutation initially described by Rall et al. (1983). He typed as an E3/4 on the Hha 1 system (W.J.S. de Villiers, personal communication). SSCP analysis of this patient (Fig. 2B.3) demonstrated that this mutation created a pattern distinct from those of the 3 common alleles and the Arg_{145}-Cys mutation. Since two of the bands from this

subject's DNA were the same as those from the E4/4 PCR product, one could conclude that this mutation occurred on an E3 background allele. The examples in Fig. 2B.2 and 2B.3 can be extended in relevant situations for rapid screening of selected patients for these mutations.

The SSCP technique has been refined and a number of modifications have been attempted in order to increase the sensitivity of the system. Kovar and co-workers (1991) developed a two-dimensional system for mutation analysis of long DNA fragments. They first restricted the desired fragment with a frequent-cutter restriction endonuclease and separated the resultant fragments under denaturing conditions in the 1st dimension. The single-stranded fragments were then electrophoresed in a 2nd dimension on non-denaturing gels to determine their single-stranded conformations.

This approach can be simplified when dealing with PCR products that are larger than the 100-200 b.p. that are optimal for SSCP, by digesting the PCR product with a restriction enzyme that results in fragments of a size more appropriate for SSCP analysis (Iwanhara et al. 1992).

Another approach, that allows the analysis of many different regions of a gene concurrently, is to first amplify the different areas simultaneously with a multiplex PCR and then to electrophorese on an SSCP system (Runnebaum et al. 1991). This method is attractive, but it can be difficult to

optimize conditions for multiplex PCR so that each of the products amplify purely and to a similar extent. In addition, the SSCP patterns of the different PCR products must not overlap.

Another group used 12 % minigels and silver-staining of DNA (non-isotopic) to detect mutations causing phenylketonuria (Dockhorn-Dworniczak et al. 1991). Ethidium bromide staining of SSCP gels on standard electrophoresis equipment has also been reported (Yap and McGee 1992). This approach is potentially cheaper, quicker, easier and safer than the conventional systems. However, it might not be as reliable in detecting subtly different bands due to the decreased resolution on minigels and the silver staining might not be as sensitive in detecting minor bands as ^{32}P.

In summary, SSCP is easy to perform in the conventional isotopic mode on sequencing apparatus. It has limited sensitivity in differentiating heterozygotes from homozygous apo E genotypes on many systems even though the PCR product is relatively small (244 bp). Perhaps the sensitivity of a particular sequence to SSCP analysis is not only a function of its length but also of its base composition. The region of the apo E gene that was analysed is about 70% GC rich. SSCP is very easy and should be considered if close to 100% detection of mutations is not a priority. A particularly useful application of this technique is in population

screening for known mutations. Here the conditions that best distinguish between the wild-type and mutant alleles can be optimised and large numbers of patients can be screened at once (see Fig. 4.13).

CHAPTER 3

PRELIMINARY CHARACTERISATION OF 2 MUTATIONS THAT RESULT IN DECREASED LDL-RECEPTOR PROTEIN SYNTHESIS

3.1 INTRODUCTION

This chapter describes the initial work on mutations that result in decreased LDL-receptor synthesis in the 2 FH homozygotes NS (an Indian) and JL (a Pedi).

NS is an Indian boy who was diagnosed as an FH homozygote on clinical grounds. His fibroblasts express no detectable LDL-receptor protein and therefore he has a "Null" mutation.

In contrast to the South African Indian population that seems to have a high prevalence of hypercholesterolemia (Seedat et al. 1990), this problem seems to be relatively rare in the South African Blacks (Marais and Berger 1986). This can be explained by the observation that this population eats foods that are low in saturated fats and cholesterol (Marais and Berger 1986) or by a decreased frequency of mutant genes that predispose to this disorder. Alternatively, hyperlipidemias are not being recognised in the Black population.

At least 2 different FH mutations have been identified in South African Blacks (van Wingerden 1981, Leitersdorf et al. 1988). The mutation in TT, a Xhosa, has been fairly extensively characterised. He was a homo-allelic homozygote whose receptors had 2 amino acids deleted from the first

cysteine-rich repeat of the ligand-binding domain. This resulted in the non-recognition of these receptors by IgG-C7, the monoclonal antibody directed to that epitope. His receptors bound LDL with normal affinity and when his fibroblasts were maximally upregulated they bound 30% of the maximum amount of LDL bound by normal fibroblasts. This confirmed findings that the first repeat of the LDL receptor is not necessary for normal LDL recognition (van Driel et al. 1987). TT's receptors also manifested slow transport from the ER to the Golgi (Leitersdorf et al. 1988).

JL, a Pedi from Lebowa, was the first Black FH homozygote characterised in South Africa (van Wingerden 1981). He was admitted to hospital at age 19 with painless swellings on both knees that turned out to be xanthomata. Besides the presence of extensive xanthomata, corneal arci and an early systolic murmur compatible with aortic stenosis, the patient appeared healthy. JL's total cholesterol readings around the time of presentation were 6,26, 5,64 and 8,87 mmol/l (van Wingerden 1981). The normal cholesterol levels reported for Black men were 3,18 ± 1,25 (Bronte-Stewart 1985). These levels in JL are particularly low for a homozygote and are similar to those found in heterozygotes in more affluent societies (Thompson et al. 1989). The clinical diagnosis of homozygous FH was confirmed in 1985 by comparing the receptor number on JL's fibroblasts and EBV transformed lymphoblastoid cells to normal values. ^{125}I-LDL metabolism at 37°C in both cell types from JL were less than 15% normal

values, as was ^{125}I-LDL and ^{125}I-IgG C7 binding at 4°C. The low LDL-receptor status in this patient was confirmed further by the demonstration of very low ACAT stimulation by LDL in this subject's cells (Coetzee et al. 1985). This study concluded that JL's cells manifested the "receptor-defective" phenotype. The last sentence of this paper reads: "The exact nature of the mutant allele awaits clarification" (Coetzee et al. 1985). In this chapter the inability of JL's mutant alleles to synthesize LDL receptors will be discussed.

JL died suddenly in 1985 aged 24 while doing a job at a neighbour's house (H. Marabe, personal communication). The severe clinical course of this untreated FH homozygote possibly illustrates an important clinical lesson: In spite of only moderately elevated cholesterol levels (6,5 - 8,9) more consistent with those of a FH heterozygote, this FH homozygote still manifested early xanthomata, an aortic stenosis murmur and premature sudden death (probably an MI). Presumably, this implies that cholesterol levels in Black homozygotes, even if partially normalised by a prudent diet and other genetic factors (Vermaak et al. 1991), are not necessarily a reliable predictive factor for the critical pathology of FH homozygotes, coronary artery disease.

3.2 RESULTS: NS

3.2.1 Clinical Presentation

NS presented at the age of 4 years with the characteristic clinical picture of an FH homozygote (Fig. 3.1). Studies of

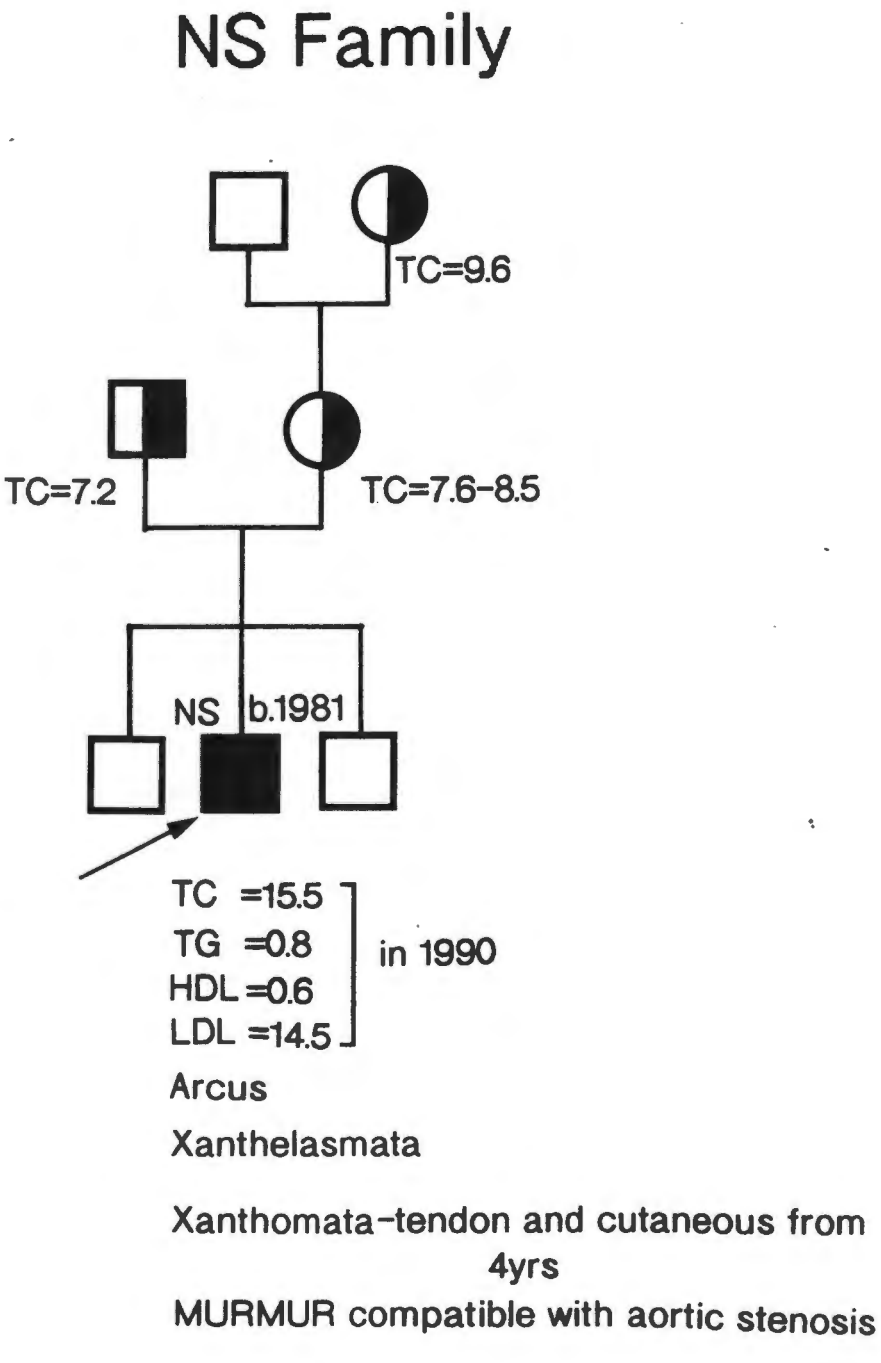


Fig. 3.1: Family tree and clinical features of NS.
TC = Total cholesterol; TG = Triglycerides;
HDL = HDL-cholesterol; LDL = LDL cholesterol;
all measured in mmol/l.

the behaviour of LDL receptors in his fibroblasts were undertaken in order to confirm the diagnosis of FH and characterise the type of mutation.

3.2.2 Ligand Binding to NS Fibroblasts

In order to determine the size of the pool of LDL receptors on his fibroblasts, ligand binding to these receptors was determined at 4°C. LDL-receptor specific binding was determined in each case by subtracting binding values obtained either in down-regulated cells or in cells simultaneously incubated with excess unlabelled ligand from total binding. No receptors were detected above background levels by the anti-LDL receptor monoclonal antibodies IgG-C7 or IgG- HL1 (Table 3.1). IgG-C7 and IgG-HL1 have distinct epitopes in ligand binding repeat 1 and the hinge-region between ligand binding repeats 4 and 5, respectively.

These findings were confirmed by more sensitive assays for LDL and ß-VLDL metabolism at 37°C (Table 3.2). The ^{125}I-LDL-bound, intracellular or degraded after 4 hours was <5% of normal values as was the amount of ^{125}I-ß-VLDL degraded (Table 3.2). LDL binding at 37°C was examined by using the dI-LDL technique. This fluorescent ligand serves as a very sensitive means of detecting LDL-receptor activity in cells. Even small amounts of dI-LDL when trapped in lysosomes give off a detectable fluorescent signal. While this was clearly evident in normal fibroblasts, no fluorescence was detected in NS fibroblasts.

Table 3.1: ^{125}I-IgG-C7 and ^{125}I-IgG-HL1 binding to NS fibroblasts at 4°C

Antibody binding to fibroblasts was performed at 0.9 - 1 μg/ml ^{125}I-IgG-C7 and 2 - 2.5 μg/ml ^{125}I-IgG-HL1 as described in general methods. Non-specific binding was determined in cells down-regulated with 1 or 2 μg/ml 25-OH cholesterol (2) or in cells to which 50 μg/ml unlabelled Ig-G-C7 was added to the cells along with the labelled antibody (1). (3) Values expressed as mean ± S.D. of 3 experiments.

Cell strain	^{125}I-IgG-C7 (ng/mg cell protein)	^{125}I-IgG-HL1 (ng/mg cell protein)
Normal	48±21 [3]	64
	(% normal binding)	(% normal binding)
NS	1.9 [2]	0 [2]
	3 [2]	7 [2]
	0 [1]	
	1.6±1.5 [3]	3.5

Table 3.2: ^{125}I-LDL and ^{125}I-β-VLDL metabolism in NS fibroblasts at 37°C

^{125}I-LDL and ^{125}I-β-VLDL metabolism was determined in fibroblasts as described in the general methods. Non-specific binding was either determined in cells down-regulated with 1 μg/ml 25-OH cholesterol [2] or in cells to which an excess of unlabelled LDL (200 μg/ml or 300 μg/ml) or β-VLDL (100 μg/ml) was added along with the labelled ligand (10 μg/ml) [1]. [3] Values expressed as mean ± standard deviation of 4 experiments.

Cell strain	^{125}I-LDL			^{125}I-β-VLDL
	Bound	Intracellular	Degraded	Degraded
	ng/mg cell protein			
Normal	233±164[3]	1319±557[3]	4511±2764[3]	1820±909[3]
	% normal			
NS	0[2]	0[2]	1[2]	3[2]
	0[1]	4[1]	0.3[1]	3[1]
	4.2[1]	7[1]	2.9[1]	0[1]
	6[1]	4[1]	1.5[1]	2.5[1]
	2.6±3[3]	3.8±2.9[3]	1.4±1.1[3]	2.1±1.4[3]

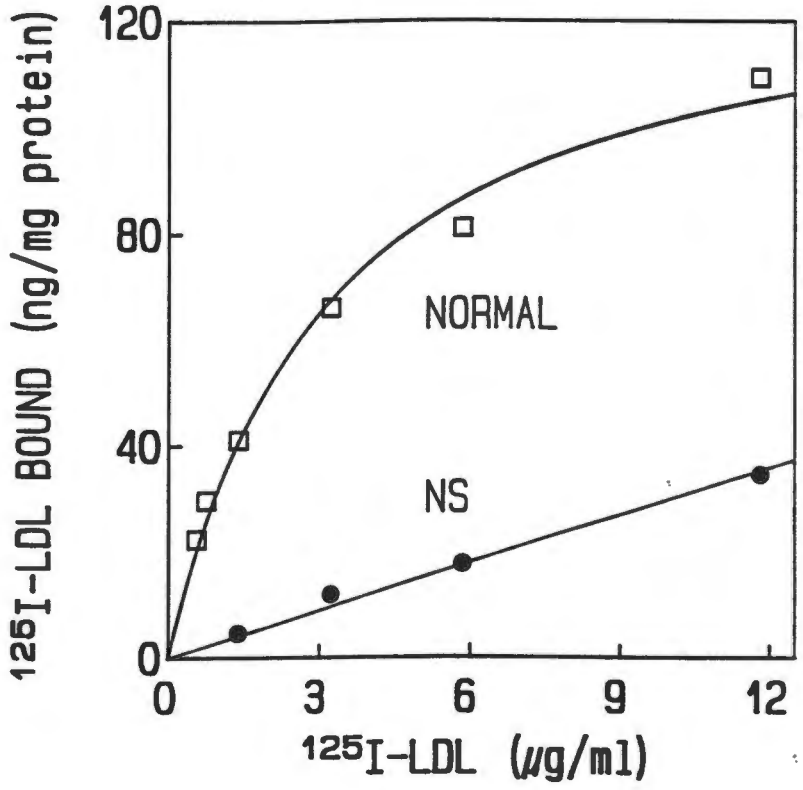


Fig. 3.2: 4°C binding of ^{125}I-LDL to Normal and NS fibroblasts. Upregulated cells were incubated at 4°C for 2 hours and the total ^{125}I-LDL bound was determined as described in the general methods. The normal cells bound ^{125}I-LDL with a B_{max} of 78 ng/mg protein and a K_d of 1.5 µg/ml as determined using commercial software (Enzfitter, Elsevier Biosoft) using one site of specific binding and non-specific processes. NS fibroblasts yielded a linear relationship of total ligand bound vs. ligand concentration that suggested predominantly non-saturable (i.e. non-receptor) binding. Each point represents the mean value obtained from duplicate dishes.

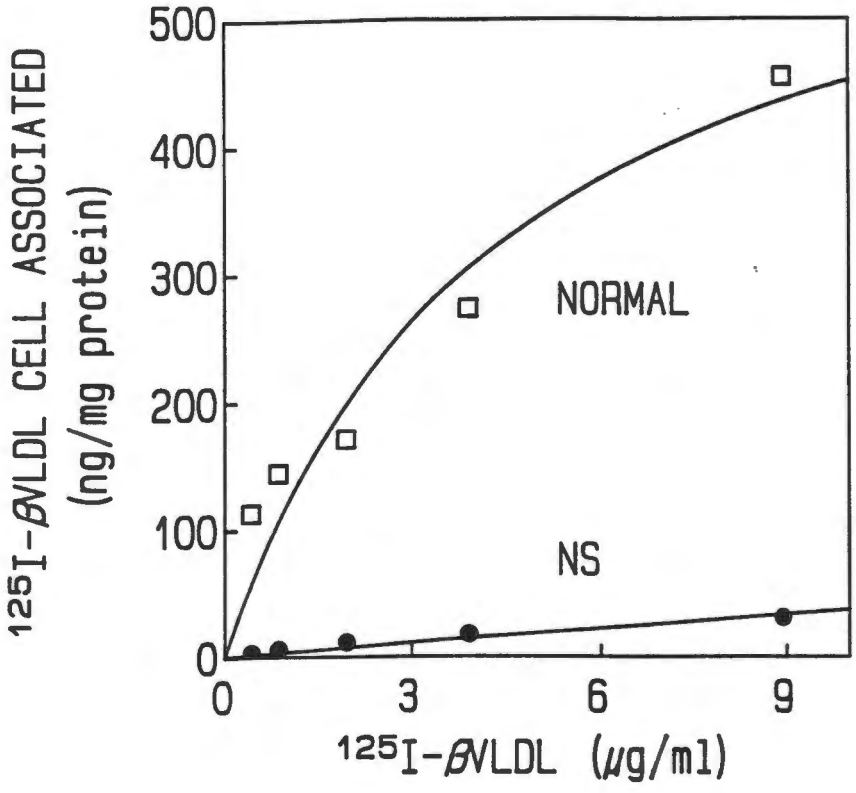


Fig. 3.3: Cell associated ß-VLDL determined at 37°C for 30 minutes in normal and NS fibroblasts. Upregulated cells were incubated for 30 minutes with the indicated ^{125}I-ßVLDL concentrations at 37°C. The cells were then washed 4x with PBS-albumin and 3x with PBS at 4°C. 0.6 ml PBS were added to the dishes and the cells were scraped and collected in microfuge tubes. This process was repeated on each dish to ensure that most of the cells were collected. The cells were centrifuged at 12 000 g for 2 minutes. The supernatant was discarded and the pellet was counted. The normal cells "bound" ^{125}I- ß-VLDL with a B_{max} of 117 ng/mg protein and a K_d of 0.08 μg/ml. NS fibroblasts yielded a predominantly linear relationship of cell-associated ligand to ligand concentration that suggested that the binding was predominantly a non-specific (non-receptor) process. (Total binding was analysed using commercial software as described in general methods.) Each point is the mean value from duplicate dishes.

An outside chance remained that the low binding values were the result of the putative mutant receptors having an impaired affinity for their ligands. This possibility was excluded in Fig. 3.2 and Fig. 3.3 that demonstrated no specific LDL binding or ß-VLDL association with NS fibroblasts in the face of high-affinity processes occurring in the normal control cells.

3.2.3 Immunoprecipitation of NS Receptors

As no receptors were detectable on NS fibroblasts we had to make the distinction between 3 scenarios: mutations that synthesise no detectable receptors (Class 1 mutations), those where there is a complete block in receptor processing (Class 2A mutations) or mutations that result in secretion of receptors (that can be placed in Class 4). The absence of any immunoprecipitable receptors in NS cells after metabolic labelling fibroblasts for 1 hour or after a subsequent chase for 5.5 hours suggested that NS harboured a "Null" mutation that resulted in no detectable protein synthesis (Fig. 3.4). Since these assays might not detect less than 5% of normal LDL receptor synthesis the distinction between very low and completely absent LDL receptor synthesis in NS cannot be made.

3.2.4 mRNA Levels in NS

"Null" mutations can result from defects at many different levels. The analysis of mRNA levels and mRNA size can help eliminate some of the possibilities. The mRNA levels in NS seemed to be very low in comparison to normal on Northern Blot analysis but the transcript was approximately the correct size (Fig. 3.5). There was also some evidence that mRNA levels in NS were regulated by sterols (Fig. 3.5). The

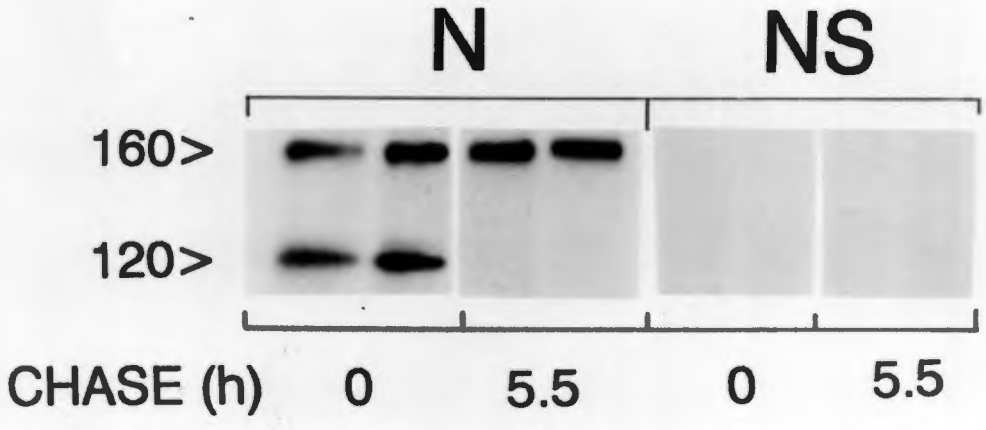
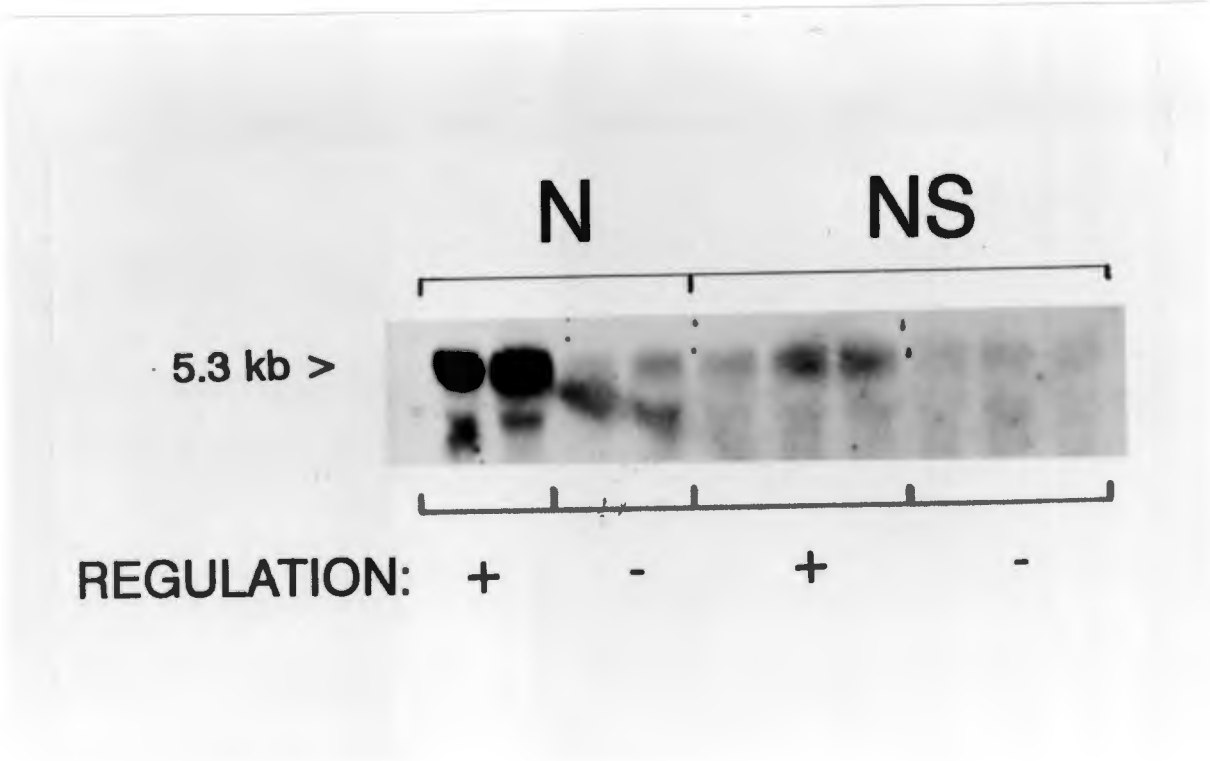


Fig. 3.4: Autoradiographs of immunoprecipitated [^{35}S]-labelled LDL receptors from normal and NS fibroblasts. Up-regulated fibroblasts were pulse labelled with [^{35}S]-methionine for 1 hour and chased in DMEM/LPDS for the indicated times. LDL-receptors were immunoprecipitated and visualised on autoradiographs as indicated in the methods. The apparent molecular weights of the precursor (120 kDa) and mature (160 kDa) receptors are indicated. The absence of immunoprecipitable LDL receptors in NS was confirmed in another similar experiment.

Fig. 3.5: Northern Blot of LDL-receptor mRNA from normal and NS cells. Cells were grown for 5 days in Dulbecco's MEM containing 10% fetal calf serum (DMEM/FCS). The cells marked + were then upregulated for 1 day by changing the medium to DMEM containing lipoprotein deficient serum (5 mg protein/ml). The cells marked - were grown for all 6 days in DMEM/FCS. 30 µg of total RNA was electrophoresed in each lane of a 1% agarose gel containing 1xMOPS and 1% formaldehyde. The RNA was blotted onto HYBOND-N and hybridised with a probe to the LDL-receptor as described in general methods and X-ray film was exposed to this membrane.



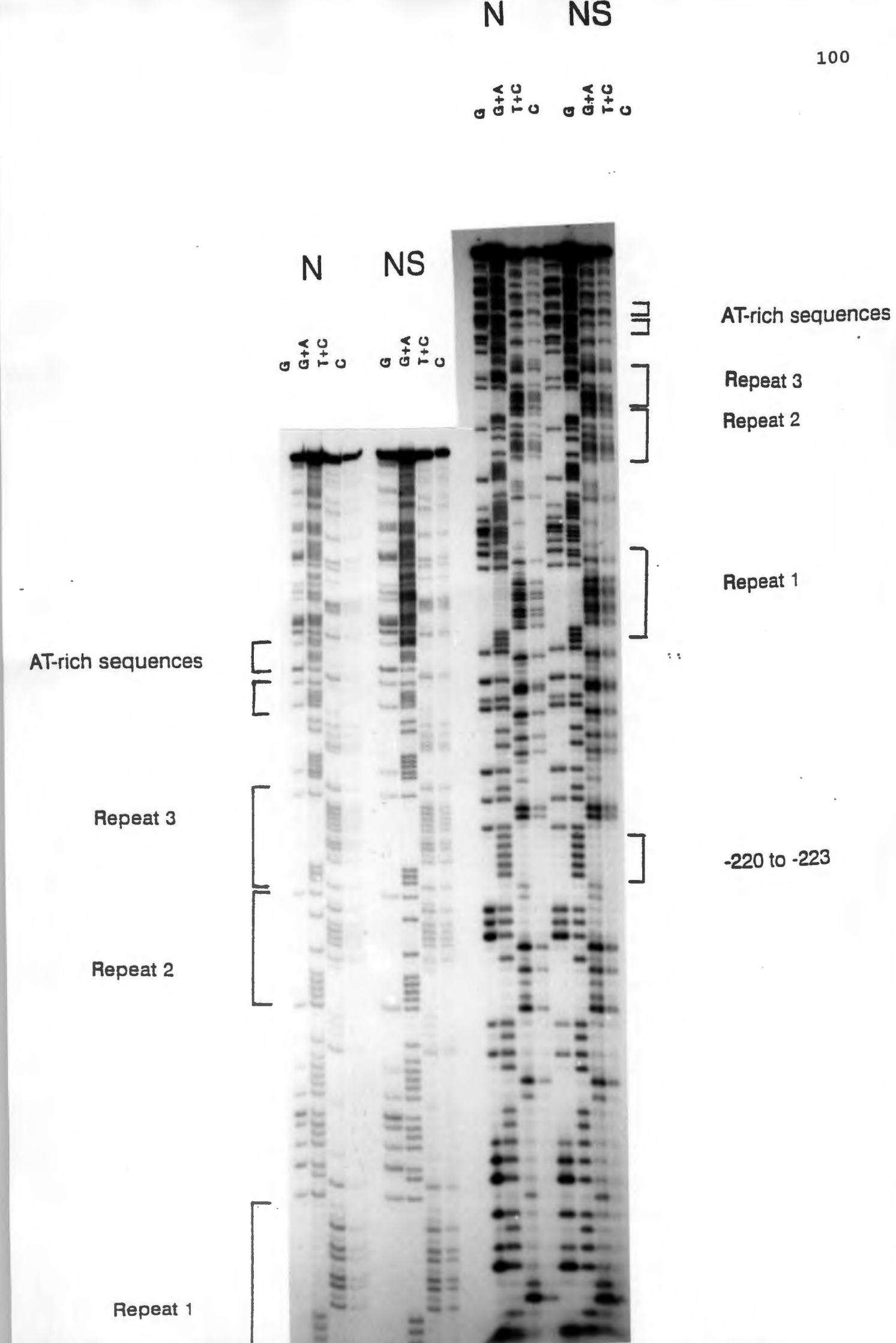
size of NS mRNA has been confirmed to be grossly normal by comparing the size of PCR products of overlapping regions of the cDNA that encompass the translated message with normal (results not shown).

3.2.5 LDL-receptor haplotype of NS

Haplotype analysis was then undertaken to determine whether NS was maybe a heteroallelic homozygote. NS was homozygous at the 4 sites examined (Ava II++, Taq 1--, Stu I++, Nco I++). This result is consistent with NS being a true homozygote. It is, however, not an absolute result since the heterozygosity index of these sites is not 100%. The 10 RFLP sites that are presently most informative only have a heterozygosity index of 86% (Hobbs et al. 1990).

3.2.6 Promoter Sequence of NS

Low mRNA levels are either the result of impaired transcription or due to message instability. The promoter region of NS' DNA was sequenced and was normal from the translation start site upstream to beyond the first Sp1 binding repeat (Fig. 3.6). This region includes all the regions of the promoter thought to be necessary for adequate transcription. NS did not have the mutations that we have detected thus far in South African Indians (Results not shown). The exact nature of the mutation in NS has not yet been characterised at the DNA level, as only limited sequencing has been performed outside the promoter region.


N
NS
G
G+A
T+C
C
AT-rich sequences
Repeat 3
Repeat 2
Repeat 1
-220 to -223

3.3 RESULTS: JL

3.3.1 Ligand Binding to JL Fibroblasts

We aimed to determine the mechanisms underlying the decreased surface expression of LDL receptors in JL's cells. It was possible that the low IgG-C7 and LDL binding previously reported in JL's cells (Coetzee et al. 1981) was due to mutations that affected the receptor's ability to recognise these ligands effectively. The low surface receptor number was confirmed by the similarly low levels of IgG-C7 and ß-VLDL that bound to JL's fibroblasts at 4°C (Table 3.3).

3.3.2 Processing of JL's LDL receptors

The biosynthesis and processing of receptors in JL's cells was examined next (Fig. 3.7). JL's cells synthesised precursor and mature receptors of normal size. The conversion of precursor to mature receptors proceeded at the normal rate with complete conversion of precursor to mature receptors within a half-hour chase period. In addition, the expression of JL's receptors was appropriately down-regulated by sterols (Fig. 3.7). JL's cells produced very few LDL receptors as judged by the amount of immunoprecipitate compared to normal cells after a 1 hour labelling period. This will be systematically investigated in 3.3.4.

3.3.3 Stability of JL's LDL receptors

A low receptor population can either be due to impaired synthesis or decreased stability of receptors. We initially examined the latter scenario. JL's cells were metabolically

Table 3.3: ^{125}I-IgG-C7 and ^{125}I-ß-VLDL binding to normal and JL fibroblasts at 4°C

Upregulated fibroblasts were incubated with 0.1 µg/ml ^{125}I-IgG-C7 with/without 50 µg/ml unlabelled Ig-G-C7 or with 3 µg/ml ^{125}I-ß-VLDL with/without 100 µg/ml unlabelled ß-VLDL. Specific binding was determined by subtracting the binding obtained in the presence of unlabelled ligand from the total binding. Values are the average of duplicate incubations.

	^{125}I-IgG-C7 bound (ng/mg cell protein)	^{125}I-ß-VLDL bound (ng/mg cell protein)
NORMAL	280	234
JL	15	27

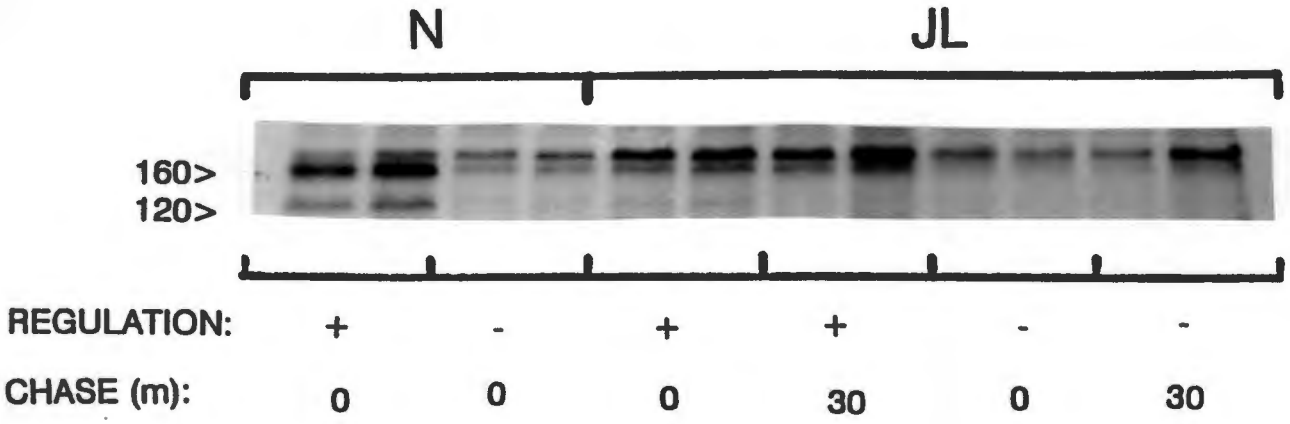


Fig. 3.7: Autoradiographs of immunoprecipitated [^{35}S]-labeled LDL receptors from normal and JL fibroblasts. Normal (N) and JL fibroblasts were grown for 5 days in Dulbecco's MEM containing 10% fetal calf serum. The cells were then upregulated by changing the medium to Dulbecco's MEM containing lipoprotein deficient serum (5 mg protein/ml) for 24 hours. The cells were then either upregulated for a further 24 hours (+) or were downregulated (-) by adding 12 ug/ml cholesterol and 1 ug/ml 25-OH cholesterol to the upregulation medium. The cells were pulsed with [^{35}S]-methionine for 1 h and were chased for the indicated times. The LDL receptors were immunoprecipitated and visualised on autoradiographs as described in general methods. The apparent molecular weights of the precursor (120 kDa) and mature (160 kDa) receptors are indicated. Please take care not to confuse the band of the mature LDL receptor with "band X" that runs just above 160 kDa.

labelled with [^{35}S]-methionine and were chased for different periods of time up to 10 hours. The half-life of JL's mature 160 kD receptors was not noticeably decreased and a significant pool of receptors remained after 10 hours of chase (Fig. 3.8).

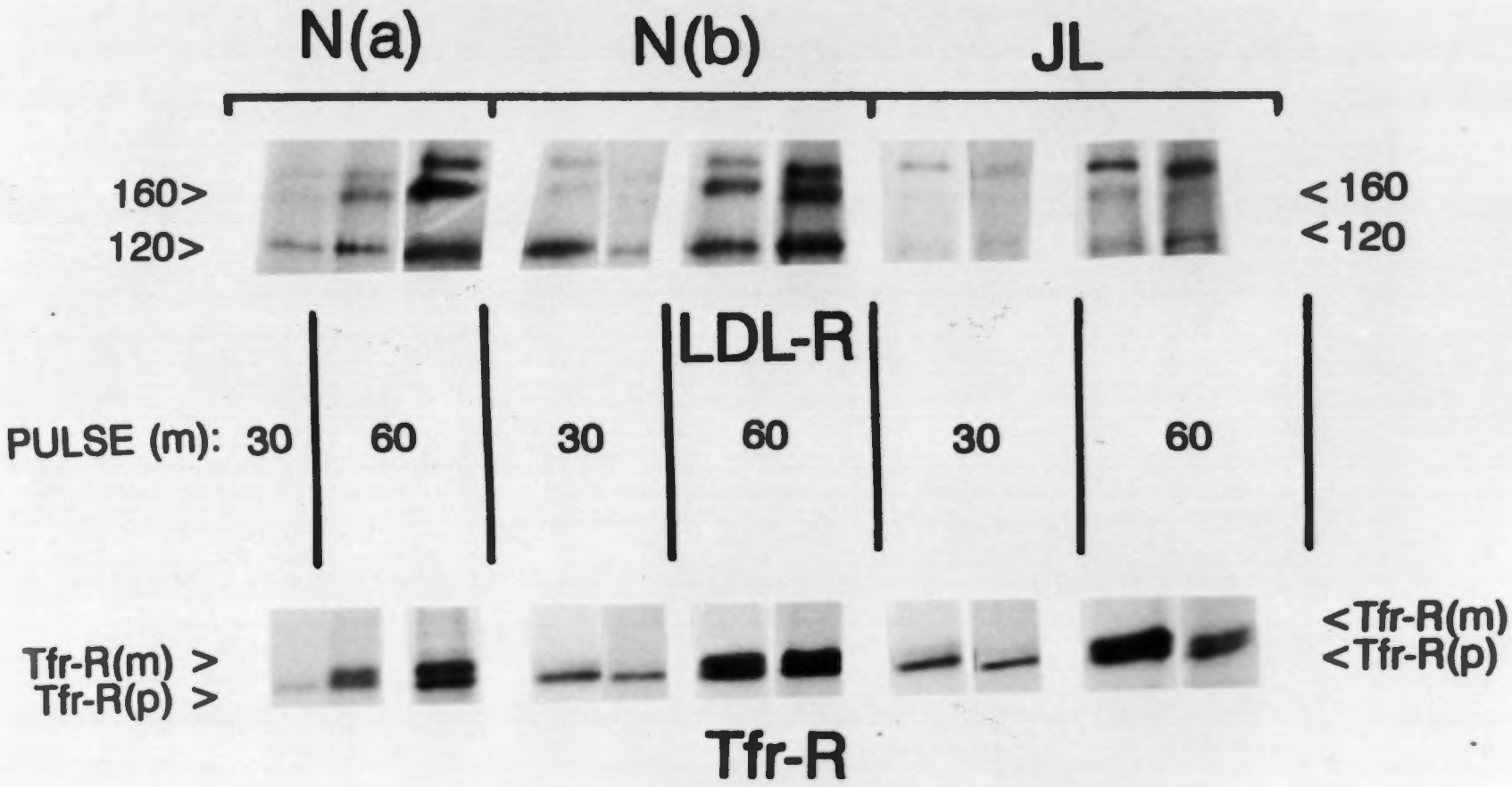
3.3.4 Synthetic Rates of JL's LDL receptors

The synthesis rates of normal and JL's receptors were determined by comparing the amount of immunoprecipitable LDL-receptor protein (mature and precursor forms), after a ½ hour and a 1 hour pulse with [^{35}S]-methionine, to total protein synthesis and/or transferrin receptor synthesis (Fig. 3.9). These synthetic rates were measured at similar cell densities. (As fibroblasts reach confluency in culture they stop dividing rapidly and their demand for cholesterol for membrane synthesis decreases. Their LDL receptor expression decreases in concert.) In each of 3 experiments where JL's receptor synthesis was determined it was decreased to below 25% of normal levels.

Impaired LDL-receptor synthesis as determined in Fig. 3.9 can either be the result of true decreased protein synthesis or due to instability of the precursor. Precursor degradation can hypothetically occur either before or after the receptor is recognisable by antibodies. If the latter case were operative, the degradation rate of these putatively unstable precursors would have to be rapid in relation to the rate of conversion to mature receptors. The total half-life of

Fig. 3.8: Half-life of LDL-receptors from normal and JL cells. Up-regulated cells were pulse-labeled with [^{35}S]-methionine for 2 hours and chased for a further 1 hour in order to convert all the LDL-receptors to the mature form. LDL receptors were then chased for the indicated times and then immunoprecipitated and visualised on autoradiographs as described in the general methods. Each time point is represented by duplicate results from both cell types. JL's receptors exhibited a normal half-life in another independent experiment.

Fig. 3.9: Synthetic rates of LDL receptors and transferrin receptors (Tfr-R) in normal and JL fibroblasts. Upregulated cells from a normal subject were grown at 2 different densities: On the day of the experiment the normal cells were either at 190 000 cells/60 mm dish (N(a)) or at 93 000 cells/60 mm dish (N(b)). JL cells were at 176 000 cells/60 mm dish. These cells were pulse-labeled for the indicated times with [^{35}S]-methionine. The LDL- and Transferrin-receptors (Tfr-R) were then successively immunoprecipitated and visualised on autoradiographs as described in the general methods. The precursor and mature forms of the LDL receptor (120 kDa and 160 kDa respectively), and of the Tfr-R (Tfr-R(p)) and Tfr-R(m)) are indicated. Similarly low LDL-receptor synthesis was observed on a number of other occasions either when compared to Tfr-R synthesis or total protein synthesis. Note that the band that appears just above the 160 kDa LDL-receptor band is a non-specific protein that has been immunoprecipitated by IgG-C7. It is not the LDL receptor as its synthesis is not affected by sterols.


N(a)
N(b)
JL
160>
120>
<160
<120
LDL-R
PULSE (m):
30
60
30
60
30
60
Tfr-R(m) >
Tfr-R(p) >
<Tfr-R(m)
<Tfr-R(p)
Tfr-R

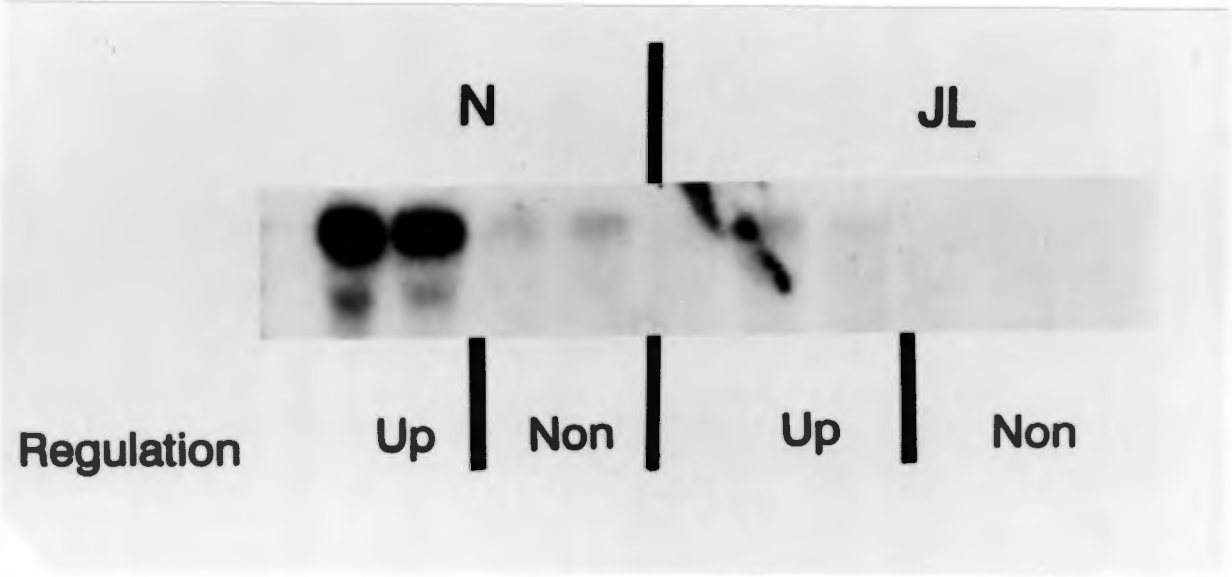


Fig. 3.10: Northern Blot of LDL-receptor mRNA from normal and JL cells. Cells were grown for 5 days in Dulbecco's MEM containing 10% fetal calf serum. The cells were either grown in the same medium for an additional day (non-regulated) or were up-regulated in Dulbecco's MEM containing lipoprotein deficient serum (5 mg protein/ml). 30 ug of total RNA was electrophoresed in each lane of a 1% agarose gel containing 1x MOPS and 1% formaldehyde. The RNA was blotted onto HYBOND-N and hybridised with a probe to the LDL-receptor as described in the general methods and X-ray film was exposed to this membrane.

precursor receptors (degradation plus maturation half-lives) would therefore be grossly decreased. Thus the ratio of precursor to mature receptors would be less than normal in JL's cells. (This is not obvious in Fig. 3.9).

3.3.5 LDL-receptor mRNA in JL's Fibroblasts

The LDL-receptor mRNA levels were then determined in JL's cells (Fig. 3.10). These low message levels could account for the decreased protein synthesis in JL's cells. The message seemed to be of approximately normal size and was regulated by sterols (Fig. 3.10).

3.3.6 DNA Sequence of JL's LDL-receptor Promoter

Analysis of the sequence of the promoter of JL's LDL receptor has yielded interesting findings (Fig. 3.11). JL is a heteroallelic homozygote and one of his alleles is missing two C's and one T in Repeat one of the promoter (G. Zuilani, H.Hobbs and L.F. de Waal personal communication). It is not possible to say exactly which three bases have been deleted. This might affect the transcription of LDL-receptor mRNA and further studies are in progress to determine what the consequences of this deletion are. The nature of the mutation in JL's other allele is not known.

Fig. 3.11: Comparison of various LDL-Receptor promoter sequences with the GC box consensus sequence

1	GC BOX CONSENSUS	: 5' G C C C A T T C C G C C C A
1	NORMAL HUMAN REPEAT 1	: 5' A A A C T C C T C C T C T T G C
2	JL REPEAT 1	: 5' A A A C T C C T C T T G C
3	XENOPUS 1 REPEAT 1	: 5' T A G C T C C T C C C T G C T G
3	XENOPUS 2 REPEAT 1	: 5' T A G C T C C T C C C C T G C C
4	HAMSTER REPEAT 1	: 5' A A G C T C C T C C C C G G C C
4	RAT REPEAT 1	: 5' A A G C T C C T C C C C G C A C
1	NORMAL HUMAN REPEAT 2	: 5' A A A A T C A C C C C A C T G C
1	MUTANT -203 TO -194	: 5' G C G C T C C T C C T C T T G C
1	MUTANT -195 TO -186	: 5' A C C T A T G C A T G C T T G C
1	MUTANT -186 TO -177	: A A A C T C C T C C G T G C A T

Footnote:

1 Sequences from Südhof et al. (1987); Mutant sequences were from constructs described in the paper. The activity of those constructs is represented in Fig. 3.12.

2 G. Zuilani, H. Hobbs and L.F. de Waal, personal communication

3 Mehta et al. (1991)

4 Bishop (1992)

3.4 DISCUSSION

In this chapter the phenotypes of the LDL receptors produced by 2 FH homozygotes have been characterised as defects of LDL-receptor protein synthesis.

3.4.1 NS

NS presented with a "Null" mutation(s) that resulted in no detectable LDL receptor synthesis. His mRNA was present at very low steady state levels but showed no evidence of any major rearrangements.

"Null" mutations with low levels of mRNA often result from the following types of defects:

1. Very large deletions generally involving the whole promoter region (e.g. French Canadian major founder mutation (Hobbs et al. 1987) result in no detectable mRNA being synthesized.
2. Mutations that create premature termination codons very often have decreased message levels (Hobbs et al. 1990). This is thought to be due to lack of ribosomal binding to a large stretch of mRNA that predisposes the message to degradation (Trecartin et al. 1981). Nonsense point mutations frequently result in decreased amounts of normal-sized mRNA. Alternatively, low levels of abnormal sized mRNA can arise from premature termination codons being created by deletions or splicing errors (Hobbs et al. 1990). An example of such a mutation is the single b.p. deletion in codon 743 in exon 15 that introduces a stop at the adjacent codon (Webb

et al. 1992). It is likely that the mutation in NS resulted from a nonsense point mutation or a small deletion that created a termination codon, since the low level of mRNA was of approximately normal size and the promoter sequence was normal.

3.4.2: JL

JL, a Pedi homozygote, had an early death presumably from a heart attack and extensive xanthomata, in spite of lower than expected cholesterol levels. Detailed discussion of this phenomenon is not possible as, to our knowledge, no autopsy was performed and other important parameters such as JL's apo E genotype and HDL cholesterol level were not available. Unfortunately JL died before simvastatin was introduced in South Africa. The mutation(s) in JL present with a very interesting phenomenon. JL's fibroblasts have low LDL-receptor mRNA levels and synthesise receptor protein at low but detectable rates. This implies that the low synthetic rate is the result of decreased but partially effective transcription. Despite this low transcription rate, LDL-receptor mRNA levels and receptor synthesis are regulated by sterols. Virtually all of the Class 1 mutations that have been described result in complete abolition of LDL-R synthesis (Hobbs et al. 1990). It is possible that the control of LDL receptor synthesis in the liver might be different to that in fibroblasts. Such a difference might contribute to the lower than expected total cholesterol level found in JL. JL's one LDL-receptor allele has a 3 bp

deletion in the first Sp1-binding repeat of the promoter (G.Zuilani, H.Hobbs and L.F. de Waal, personal communication). The sequence of the promoter of JL's other allele is normal. The three direct repeats in the LDL-receptor promoter each include a 10 bp sequence homologous to the GC-box sequence (see Fig. 3.11) that is thought to be the consensus sequence for the transcription factor Sp1 (Kadonaga 1986). Repeats 1 and 3 that deviate from this consensus sequence at the 2 and 1 sites respectively, both bind Sp1 in vitro (Dawson et al. 1988). However, repeat 2 that deviates at 3 sites does not bind Sp1. The sequences of repeat 1 in Xenopus, the hamster and the rat never deviate by more than 2 bp from the consensus sequence (Fig 3.11). The sequence of JL that harbours the deletion deviates by four bp from the consensus sequence and it is thus possible that this results in impaired Sp1 binding leading to decreased transcription. In vitro mutagenesis experiments on the LDL-receptor promoter region have been performed by Südhof et al. (1987). A vector was constructed that contained the LDL-receptor promoter region linked to the coding region of the bacterial chloramphenicol acetyltransferase gene. Each of the fifteen mutations that were created in the LDL-receptor promoter region of this vector had a cassette of 10 base pairs that was scrambled. These cassettes of mutations overlapped and spanned the three imperfect direct repeats and the TATA like sequences. A summary of the results is shown in Fig. 3.12. The mutations that pertain to repeat 1 are shown in Fig. 3.11. These experiments suggest that mutations in repeat 1

affect transcription less severely (50% - 90%) than mutations in the other 2 repeats. It is also interesting that transcription is not abolished when bp -195 to -186, that comprise almost the entire Sp1 consensus sequence (Fig. 12) of repeat 1, are scrambled. When only 3 bp (bp -203 to -194 (see Fig. 3.11)) were scrambled a significant reduction of transcription occurred (Fig. 3.12) suggesting that the entire repeat and not only the consensus sequence is probably recognised by the transcription factor (Südhof et al. 1987). These experiments suggest that transcription of the LDL-R gene is sensitive to mutations in repeat 1. However it would seem that mutations in repeat 1 do not entirely abolish transcription. These experiments did not address the effects of deletions in repeat 1. Studies are currently being pursued to determine whether the deletion in JL affects transcription, possibly by reducing Sp1 binding.

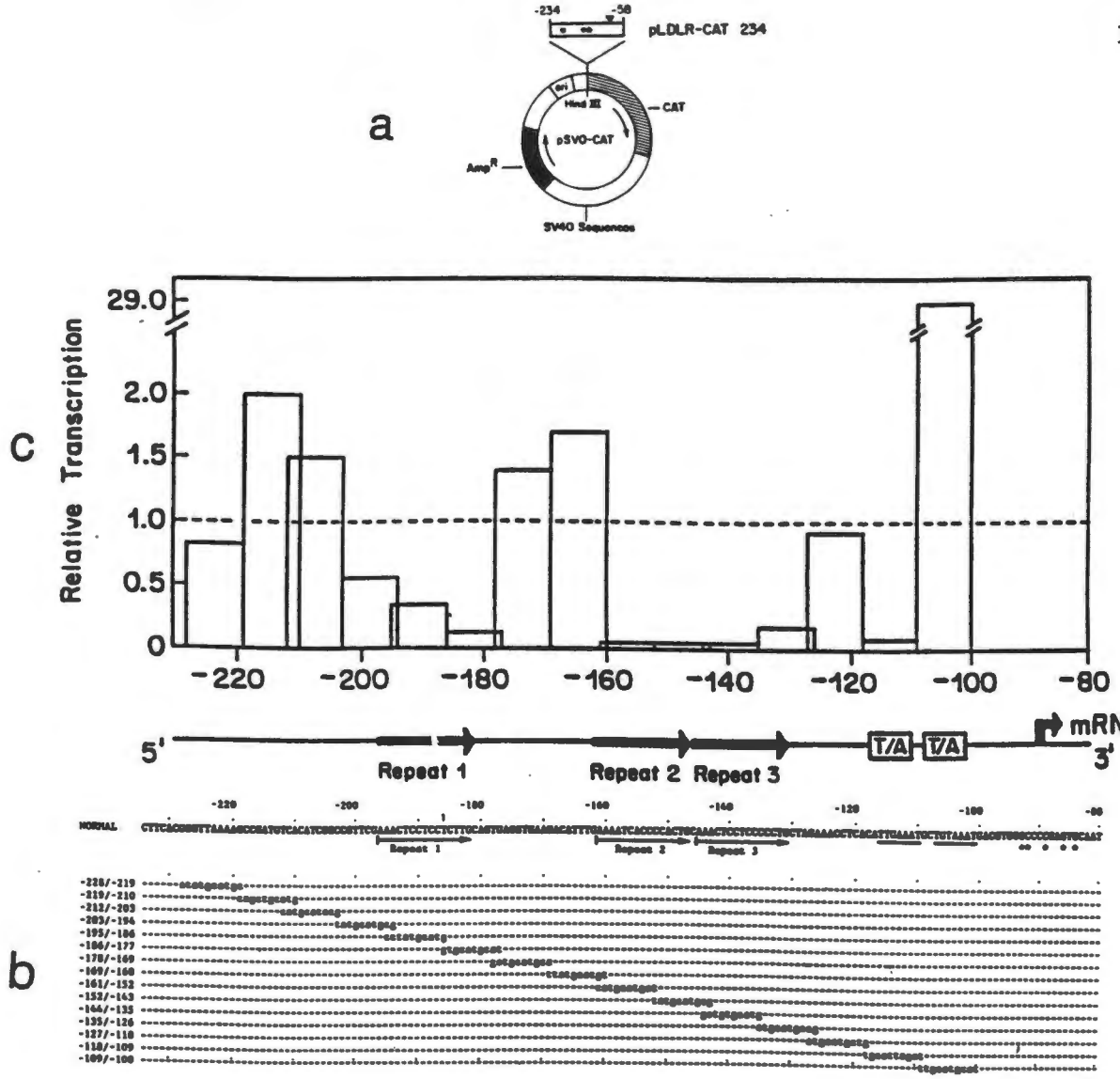


Fig. 3.12 (from SÜdhof et al., 1987)

A fragment of the human LDL-receptor promoter from -58 to -234 (the A of the ATG initiation codon is +1) was inserted into pSVO-CAT(a). The sequence of a portion of this promoter is shown at the top of (b) and the important transcriptional elements are labelled. Fifteen different overlapping mutations, where 10 b.p. were scrambled, were introduced into this plasmid. The 10-bp sequence that was scrambled is shown on the left of (b) and is shown in lower case letters below the normal sequence.

The normal and the mutated plasmids were co-transfected along with a control plasmid, pHSVTK-CAT into CHO cells (pHSVTK-CAT contains a part of the herpes simplex virus thymidine kinase promoter). The relative transcription activity of the normal and mutant LDL-receptor promoters was expressed as the ratio (x/y) of the amounts of primer extension products of the mRNAs from the transfected LDL-receptor-CAT gene (x) and from the HSVTK-CAT gene (y). The normal LDL-receptor promoter activity was normalised to the value of 1. The ratios of the activities of mutant promoters relative to the normal promoter (1) was plotted (c). The locations of the mutations are shown on the x axis (c). A diagram of the normal LDL receptor promoter is at the bottom of (c).

CHAPTER 4

THE LDL-RECEPTOR MUTATIONS IN D

4.1 INTRODUCTION

A practical prerequisite for undertaking an investigation of FH in any population is to have access to patient material. Skin fibroblasts from 4 different unrelated homozygote South Africans of Indian origin provided a starting point for the study of FH in this group. Two of the patients, AV and AA, were from the Transvaal Province and NS and D came from Pietermaritzburg and Durban, respectively, in the Natal Province of South Africa. Preliminary findings allowed us to conclude that there were at least three different LDL receptor mutations in South African Indians: NS manifested a "Null" mutation; 2. AV and AA were indistinguishable and were characterised as being receptor defective expressing about 25% the normal number of receptors on their fibroblasts; 3. D also expressed around 25% the normal number of receptors. However, in contrast to AV and AA, his receptors did not bind LDL to the same extent as ß-VLDL and IgG-C7.

Subsequently two different LDL receptor mutations were detected in D. The path taken in the discovery of the mutations in D, from the protein phenotype to the genotype, will be outlined in this chapter.

4.2 RESULTS

4.2.1 Clinical Background

The patient D was originally recognised as a FH homozygote by Dr I. Jialal. D's genogram and clinical features are shown in Fig. 4.1. It is clear that the index patient and his sibling have classic features of homozygous FH. D's father seems to have had raised lipid levels and an early death from an MI compatible with his obligate heterozygote genotype, but his mother has very low cholesterol levels for an obligate heterozygote. Unfortunately fibroblasts and DNA from D's parents were not available and contact with this family seems to have been lost.

4.2.2 Ligand Binding and Metabolism by D's LDL Receptors

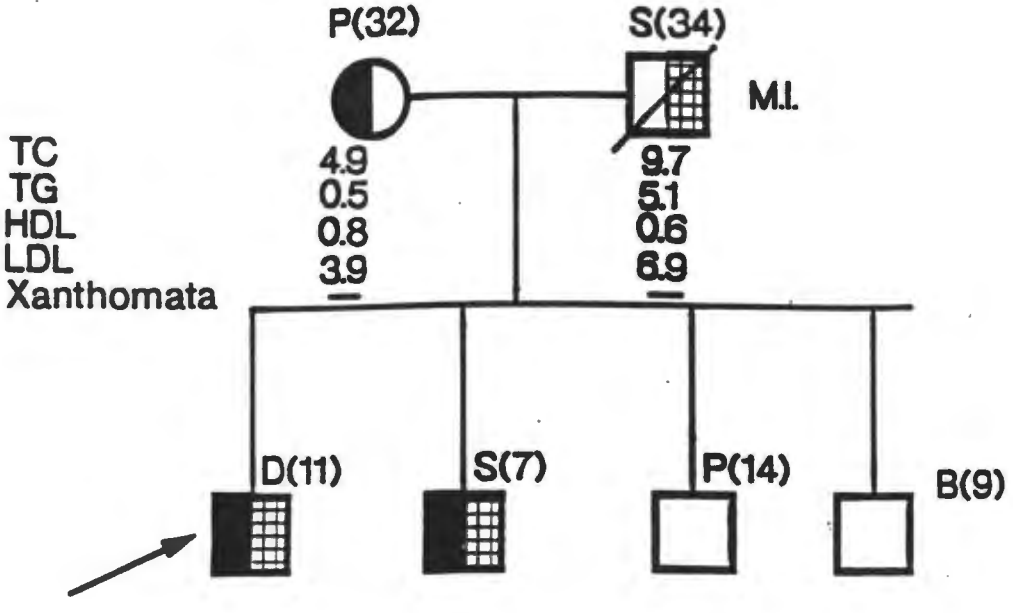
The ability of D's fibroblasts to bind and metabolise various ligands was initially examined (Table 4.1). D's upregulated fibroblasts consistently bound, internalised and degraded less than 10% of the normal amount of ^{125}I-LDL when incubated with 10 μg/ml ^{125}I-LDL for 4 hours at 37°C. However his cells seemed to express around 30% the normal number of LDL receptors as determined by their ability to bind ß-VLDL and IgG-C7 (Table 4.1). One or both of the following scenarios could explain this discrepancy between the IgG-C7 and ß-VLDL binding on the one hand and the LDL binding on the other. The receptors produced by D's mutant alleles might be expressed at about 30% the normal level on the surface of his cells but have a lower affinity for LDL. Alternatively, there might be at least two populations of receptors on his

fibroblasts: one population that recognises LDL, ß-VLDL and antibody normally and another population that has a very low affinity for LDL, but a normal affinity for the other two ligands.

Binding isotherms established that the number of receptors on D's cells determined in Table 4.1 was not a falsely low estimate due to the receptors having an impaired affinity for antibody binding. D's fibroblasts or normal cells were incubated with increasing concentrations of ^{125}I-IgG-C7 and it was determined that his cells indeed expressed about 30% the normal number of receptors that had a normal affinity for this antibody (Fig. 4.2).

The ability of D's receptors to recognise LDL was then determined by incubating normal and D's fibroblasts with varying concentrations of ^{125}I-LDL at 37°C for 4 hours (Fig. 4.3). The normal cells yielded saturable binding isotherms and were analysed using commercial software (Enzfitter Elsevier Biosoft) assuming one high-affinity binding site and non-saturable processes. The ^{125}I-LDL concentrations that resulted in half-maximal binding, internalization and degradation at 37°C in the normal cells was 11 µg/ml, 8 µg/ml and 6 µg/ml respectively. In contrast, the shape of the curve in mutant cells was a straight line implying that the predominant process that was being measured was non-saturable binding. This suggested that the affinity of D's receptors for LDL was indeed very low. These results

D Family



TC	19.5	16.9	4.7	3.5
TG	1.9	1.3	1.2	1.5
HDL	0.4	0.6	0.8	0.8
LDL	18.8	15.7	3.4	2.0
Xanthomata	+++	+++	—	—

Fig. 4.1: Family tree and clinical features of D.
TC = Total cholesterol; TG = Triglycerides;
HDL = HDL-cholesterol; LDL = LDL cholesterol; all measured in mmol/l. Xanthomata were interdigital and tendinous.

Table 4.1: 4°C ^{125}I-IgG-C7 binding and 37°C metabolism of ^{125}I-LDL and ^{125}I-β-VLDL in normal and D fibroblasts

^{125}I-ligand binding and metabolism in upregulated fibroblasts was performed with 1 μg/ml ^{125}I-IgG-C7, and 10 μg/ml ^{125}I-LDL and ^{125}I-β-VLDL as described in the Methods. Non-specific binding was determined either in cells downregulated for 48 h with 12 μg/ml cholesterol and 1 μg/ml 25-OH cholesterol[1] or in cells to which an excess of cold ligand was added along with the labelled ligand (50 μg/ml IgG-C7[2], 200 μg/ml LDL[3] and either 100 μg/ml [4] or 200 μg/ml β-VLDL[5]). In experiments 1-4 the normal values are the average of 2 normal cell types while in experiment 5 only 1 normal cell type was used. All binding values are averages of duplicate incubations expressed in ng/mg cell protein.

EXPERIMENT NUMBER	CELL TYPE	4°C ^{125}I-IgG-C7 Bound	37°C ^{125}I-LDL Bound	37°C ^{125}I-LDL Intracellular	37°C ^{125}I-LDL Degraded	37°C ^{125}I-β-VLDL Bound	37°C ^{125}I-β-VLDL Intracellular	37°C ^{125}I-β-VLDL Degraded
1	Normal	56 [1]	144 [3]	1048 [3]	1904 [3]	163 [5]	588 [5]	375 [5]
	D	11	12	82	82	29	201	90
2	Normal	51 [2]						
	D	22						
3a	Normal	46 [2]	231 [3]	1644 [3]	3595 [3]			
	D	13	0	133	302			
3b	Normal	53 [1]	242 [1]	1584 [1]	3908 [1]			
	D	15	9	114	361			
4	Normal	33 [2]	186 [3]	762 [3]	2319 [3]	84 [4]	648 [4]	824 [4]
	D	10	18	77	235	27	204	270
5	Normal		449 [3]	1319 [3]	6971 [3]			
	D		28	103	618			
Average ± SD activity in D as a % normal activity		30±8	6±5	8±1	8±2	25	34	29

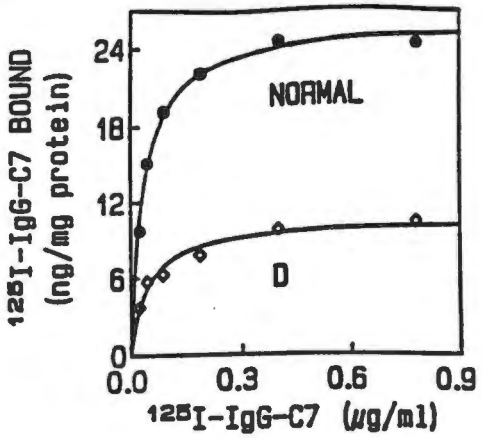


Fig. 4.2: 4°C Binding of ^{125}I-IgG-C7 to Normal and D fibroblasts. Upregulated cells were incubated at 4°C for 2 hours with varying concentrations of ^{125}I-IgG-C7 and the total ^{125}I-ligand bound was determined as described in the general methods. Each value represents the average of a duplicate incubation. Normal and D fibroblasts bound this antibody with Kd's of 0.045 ug/ml and 0.03 ug/ml respectively. The B_{max} of D's fibroblasts was 33% of the normal value.

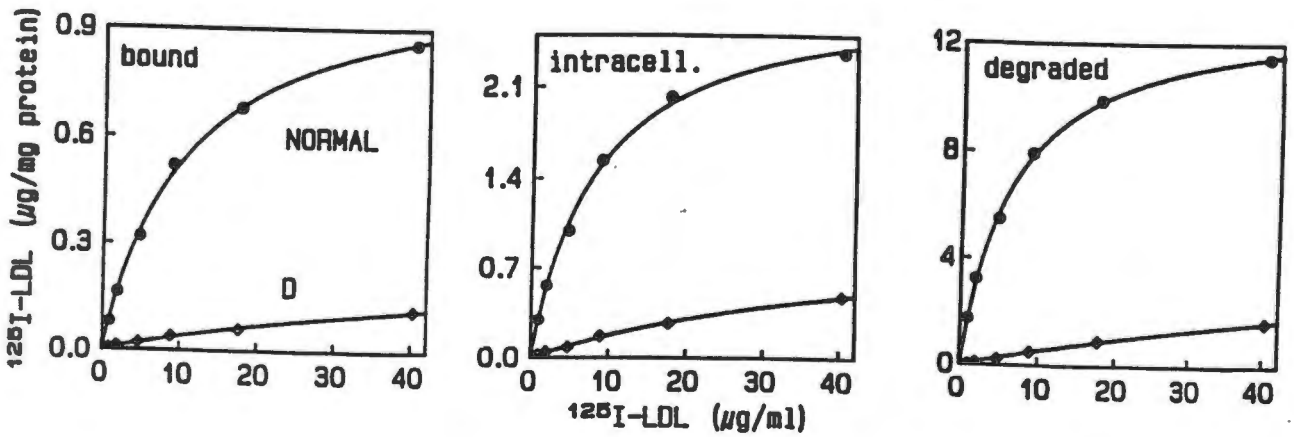


Fig. 4.3 ^{125}I-LDL metabolism in normal and D fibroblasts at 37°C. Upregulated normal and D fibroblasts were incubated with the indicated ^{125}I-LDL concentrations for 4 hours at 37°C. Then the ^{125}I-bound, intracellular and degraded were determined as described in the general methods. When these total activities were analysed using commercial software (Enzfitter, Elsevier biosoft) assuming one specific site plus non-specific processes, the normal cells yielded apparent Kd's of 11 ug/ml, 8 µg/m and 6 ug/ml for ^{125}I-LDL bound, intracellular and degraded, respectively. D's fibroblasts yielded virtual straight lines for these processes suggesting that the activity in these dishes was mainly due to non-specific (non-receptor) processes. Each point represents the average value obtained from duplicate dishes.

do not rule out the 2 population model but do suggest that if this is the case, then the proportion of receptors on D's cells that recognise LDL normally is very low.

4.2.3 LDL-Receptor Biosynthesis and Stability in D's Fibroblasts

The cause of the receptor-defective phenotype was investigated in experiments where D's cells and normal controls were metabolically labelled with [^{35}S]-methionine for an hour and then chased for varying periods. The synthesis of receptors in D's cells was determined to be grossly normal since the amount of [^{35}S]-methionine incorporated into LDL receptors as a proportion of total protein synthesis was similar in D's and normal fibroblasts (Fig. 4.4). The majority of the receptors synthesised by normal cells were of the mature type (160 kDa) after 1 hour of labelling and the residual precursor (120 kDa) receptor pool in these cells was converted to the mature form after a further 4 hours of chase (Fig. 4.4). In D's cells, however, the majority of the receptors were still in the precursor form (correct size of 120 kDa) after 1 hour of metabolic labelling (Fig. 4.4). Some of this precursor receptor pool was still present in D's cells after a further 4 hours of chase (Fig. 4.4). The mature receptors in D were of the correct size (160 kDa) (Fig. 4.4). These results suggest that there was slowed transport of D's receptors from the ER to the Golgi.

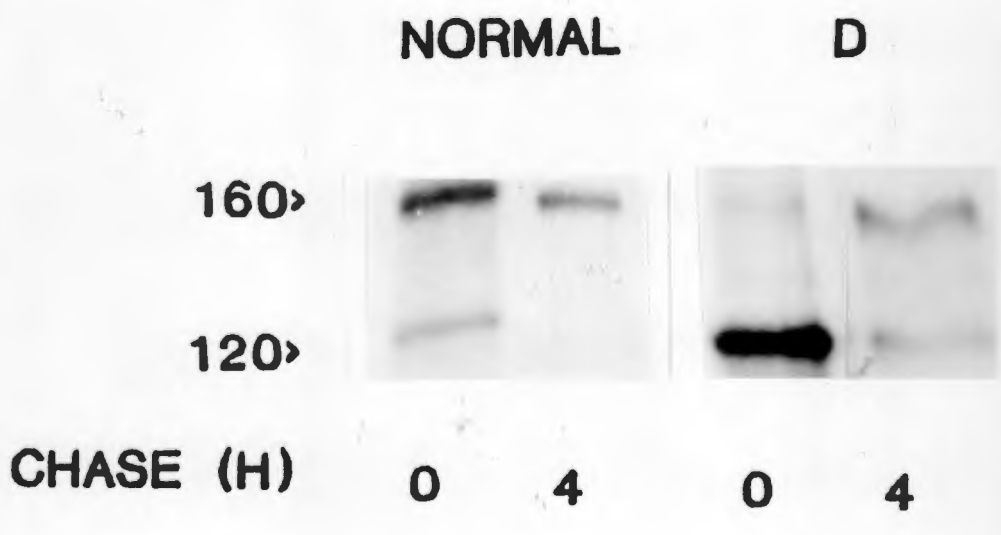


Fig. 4.4 LDL-Receptor processing in N and D cells. Upregulated fibroblasts were pulse-labeled with [^{35}S]-methionine for 1 hour and chased in DMEM/LPDS for the indicated times. LDL-receptors were immunoprecipitated and visualised on autoradiographs as indicated in the Methods. The apparent molecular weights of the precursos (120 kDa) and mature (160 kDa) receptors are shown.

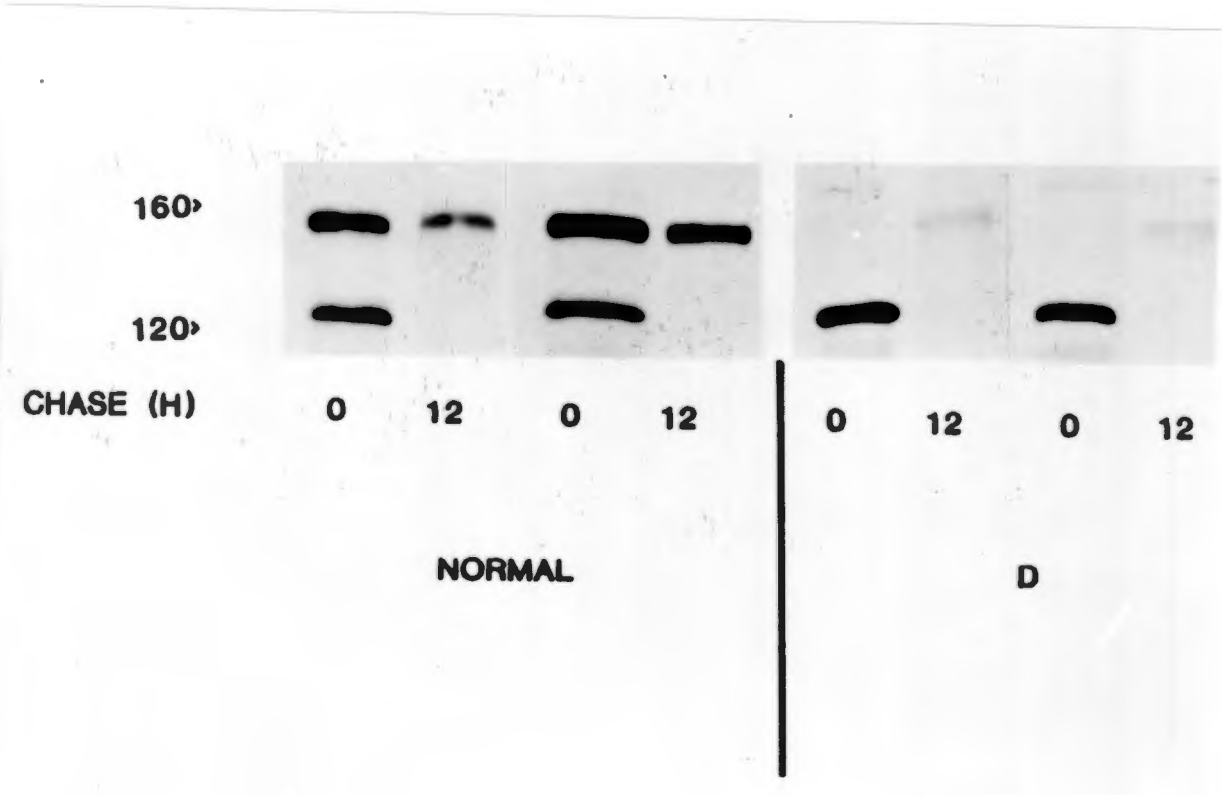


Fig. 4.5 Degradation of Normal and D's LDL-receptors. Upregulated fibroblasts were pulse-labelled with [^{35}S]-methionine for 1 hour and chased in DMEM/LPDS for the indicated times. LDL-receptors were immunoprecipitated and visualised on autoradiographs as described in the general Methods. The apparent molecular weights of the precursor (120 kDa) and mature (160 kDa) receptors are shown.

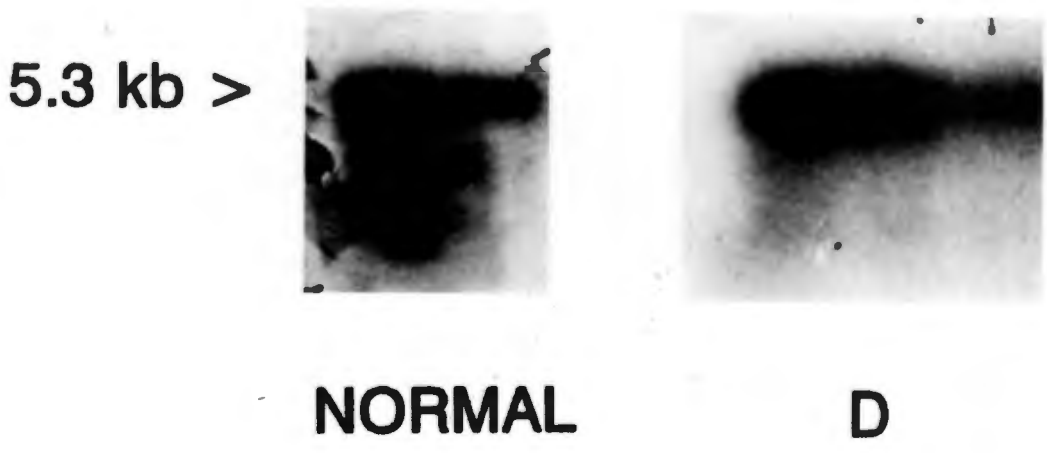


Fig. 4.6 Northern blot of LDL-receptor mRNA from normal and D fibroblasts. 30 ug of total mRNA from upregulated fibroblasts was electrophoresed in each lane of a 1% agarose gel contaning 1x MOPS and 1% formaldehyde. The RNA was blotted onto HYBOND-N and hybridised to a probe to the LDL receptor as described in the general methods. This membrane was then used for autoradiography.

The stability of D's receptors was then examined by pulsing the cells with [^{35}S]-methionine for 1 hour and then chasing them in the absence of methionine for 12 hours. The ratio of the receptors in the normal cells before and after the 12 hour chase was consistent with a normal mature receptor half-life of around 10-12 hours (Casciola et al. 1988, Knight et al. 1989). D's receptors almost completely disappeared after the 12 hour chase (Fig. 4.5). This suggested that the low steady-state level of LDL receptors in D was largely the result of their instability.

4.2.4 mRNA Levels in D

Northern Blot analysis of the mRNA in upregulated cells showed that D had normal sized transcript present at approximately normal steady state levels (Fig. 4.6).

4.2.5 The Approach to the LDL-receptor Mutations in D

Haplotype analysis of PCR products of genomic DNA determined that this subject was a heteroallelic homozygote since he was a heterozygotye at both the Ava II and Hinc II RFLP sites (results not shown). Since the mutations in D impaired LDL binding, these were sought in the ligand-binding domain and EGF-precursor homology repeat A (Esser et al. 1989, Russell et al. 1990). Overlapping PCR products obtained from first strand cDNA comprising the ligand-binding domain and part of the EGF-precursor domain were analysed on single-strand conformational polymorphism (SSCP) gels (Fig. 4.7). The band patterns obtained from the denatured PCR products spanning

Fig. 4.7 SSCP analysis of amplified fragments of normal and D cDNA. cDNA from D was prepared as described in general methods. A plasmid contaning the normal LDL-receptor cDNA, pLDL-R2, was used as the normal control. Fragments of the indicated exons of the LDL receptor were amplified by PCR (see Table 2A.1) using the appropriate oligonucleotides as shown. The PCR products were labelled by [α^{32}P]-dCTP incorporation. These fragments were either denatured in formamide (denatured: +) or run undenatured (denatured: -) on a 6% Acrylamide gel containing 10% glycerol and 1x TBE at 40W at room temperature. The gel was then used for autoradiography. Note that the bands formed in the exon 1-3 and exon 4 PCR products are different in the normal sample and in D. Improved SSCP resolution of these mutations can be found in Figures 4.13a and 4.13b.

SAMPLE :	D N	N D	D N	N D	N N D D
DENATURED:	+ +	+ +	- -	- -	+ - + -
EXON(S):	4	1+2+3	4	1+2+3	5+6+1/2 of 7
OLIGOS:	G25/G26	G13/G14	G25/G26	G13/G14	G27/G28

Fig. 4.8: LDL-receptor PCR products of cDNA made from D's mRNA. cDNA was reverse-transcribed from mRNA extracted from D's upregulated fibroblasts as described in the general methods. This cDNA was amplified in PCR reactions that contained oligonucleotides AR12 and AR13, AR14 and AR15, AR16 and AR17, AR6 and AR7, AR8 and AR9 that spanned exons 1-4, 5-8, 9-12, 13-15 and 16, 17 and part of 18 of the LDL-receptor respectively. The major bands gave the expected sizes of 718 b.p., 541 b.p., 726 b.p., 533 b.p. and 355 b.p. PCR conditions are outlined in table 2A.1 of the general methods.

exons 1 to 3 and that spanning exon 4 were different for D and the normal control (Fig. 4.7). This suggested that one of D's mutations was in exons 1-3 and the other in exon 4.

Overlapping regions of the coding region of the cDNA from D were amplified by PCR (Fig. 4.8). The one set of primers amplified exons 1-4 (lane 1). The bands were cut out from the gel and were subsequently crushed and eluted into TE. The primers for the exon 1-4 product were created with Bam HI (AR12) and Sph I (AR13) restriction sites close to their 5' ends. This allowed us to insert the PCR product into the polylinker of pUC 118 and generate clones for DNA sequencing. The main reason for opting for cloning was that direct DNA sequencing can be difficult to interpret in heteroallelic genes.

DNA sequence analysis yielded different mutations in clones 1 and 2. Clone 1 had a G-A mutation at codon 119 (GAG(Glu)-AAG(Lys)) in the ligand-binding repeat 3 in exon 4 (Fig. 4.9). Clone 2 had a G-T mutation at codon 69 (GAT(Asp)-TAT(Tyr)) in ligand-binding repeat 2 in exon 3 (Fig. 4.10). To confirm that these mutations were true mutations and not errors introduced by the PCR reaction, one or both of the following methods were used: the mutation in exon 3 was confirmed by direct sequencing of a second PCR product prepared from a second cDNA preparation (Fig. 4.11). The mutation identified in exon 4 eliminated an Mnl 1 restriction site. This was demonstrated by Mnl I restriction digestion

Fig. 4.9: DNA sequence of mutation in codon 119 of D. D's cDNA was PCR-amplified by AR12 and AR13 (see Table 2A.1), inserted into pUC 118 and sequenced with the Taq Track kit (Promega) using [τ^{32}P]-ATP-kinased AR12 (see general methods). Exons 1-4 of the normal LDL receptor cDNA sequence in pLDL-R2 was subcloned in M13mp19 by restriction digestion with Eco RI and XbaI. This was sequenced with the Taq Track Kit using 5'-^{32}P-labelled universal primer. The site of the mutation is indicated.

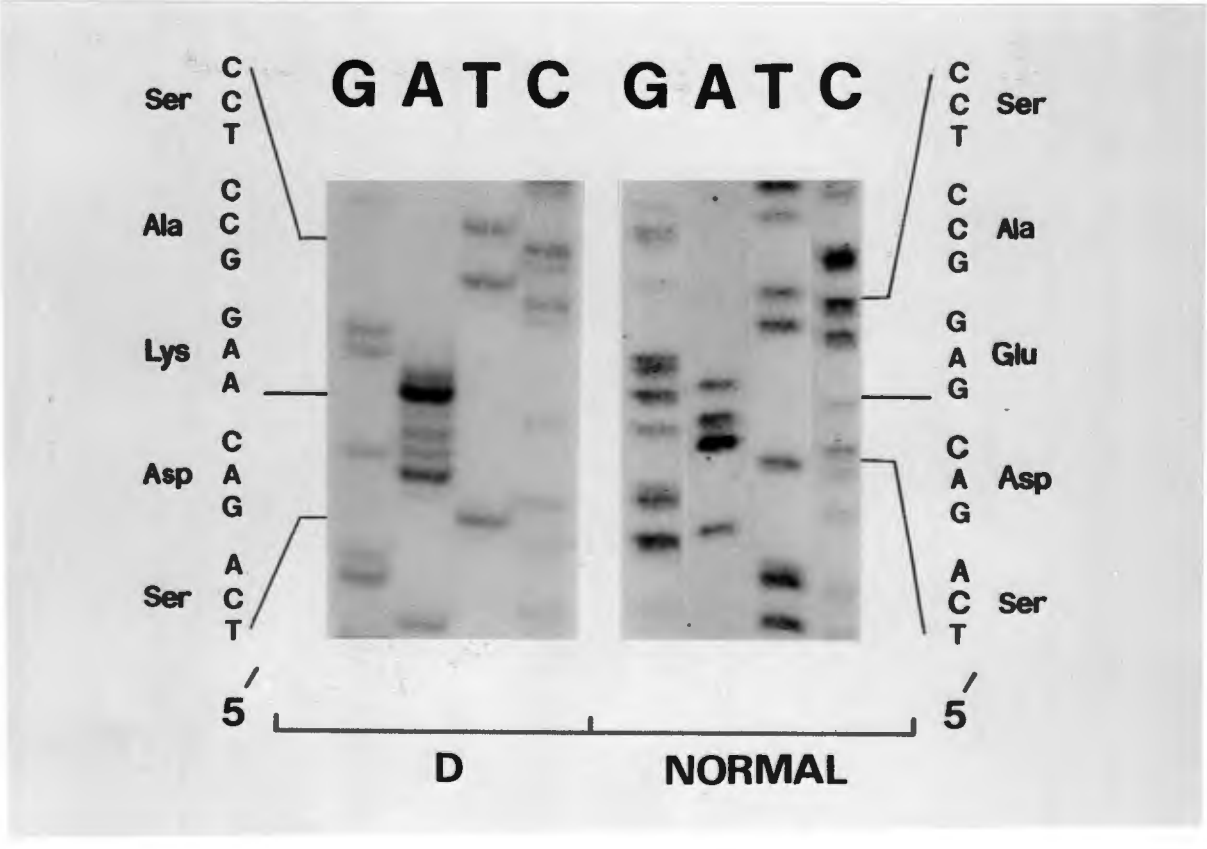
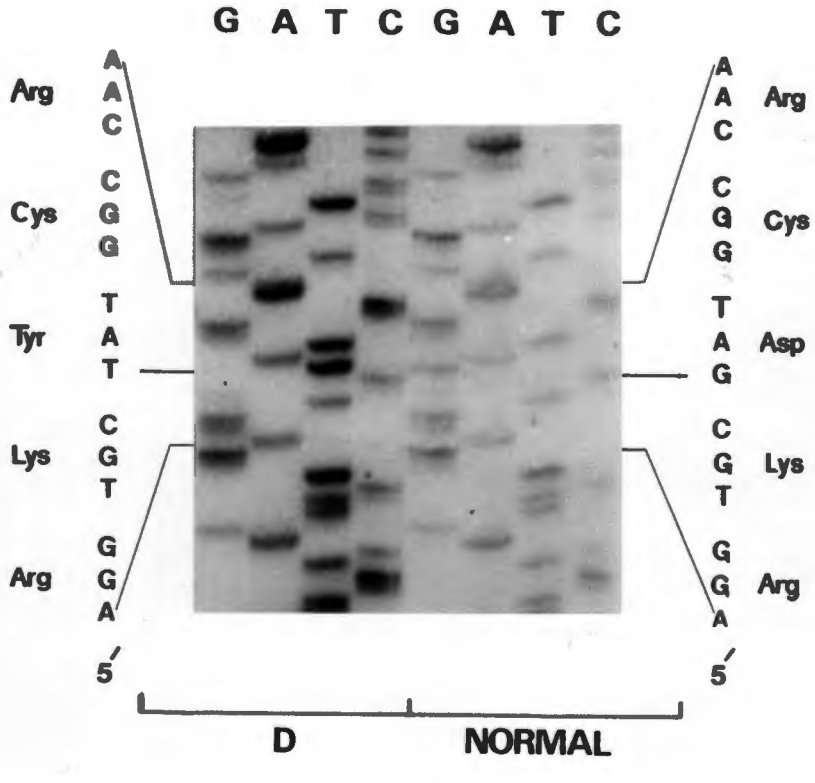


Fig. 4.10: DNA sequence of mutation in codon 69 of D's LDL-receptor. D's cDNA was PCR-amplified by AR12 and AR13 (see Table 2A.1) and inserted into pUC 118. Clones 1 (normal sequence) and 2 (D's mutant sequence) were sequenced with the TaqTrack kit using [γ-^{32}P]-ATP-kinased AR12 (see general methods). The site of the mutation (GAT to TAT) is indicated.



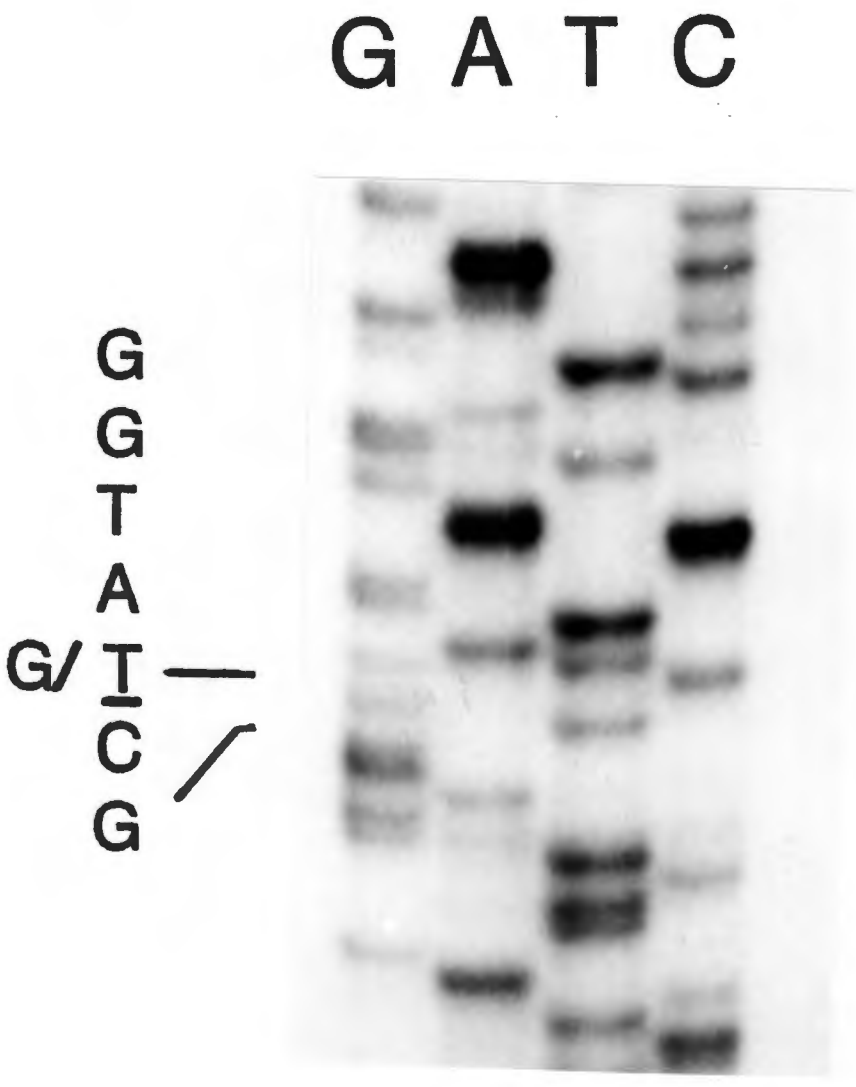
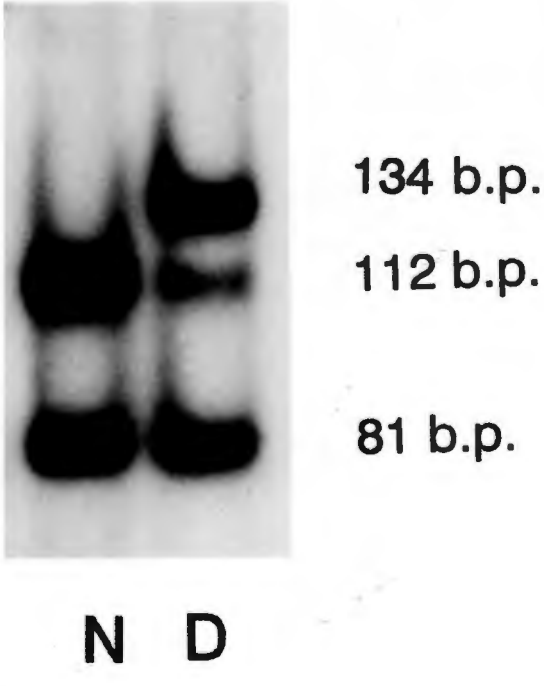


Fig. 4.11: Direct sequencing of the codon 69 mutation in D. cDNA from D was amplified using oligonucleotides AR12 and G14 using the conditions of the G13-G14 PCR (see table 2A.1). The PCR product was electrophoresed on 6% acrylamide and the correct size band was cut out and eluted. This fragment was sequenced with the fmol kit (Promega) using kinased AR12 as described in the general methods. The cDNA preparation used for this direct sequencing was made independently from the preparation that was cloned and sequenced in Fig. 4.10.

Fig. 4.12: Diagnosis of codon 119 mutation in D by Mnl I digestion. The 5' end of exon 4 of genomic DNA from a normal subject and D was amplfied by PCR using oligonucleotides G31 and 4.3 (see table 2A.1, general methods). The PCR product was radiolabelled by [α^{32}P] dCTP incorporation. This was then digested overnight with Mnl I (4u) and electrophoresed on 12% polyacrylamide. The gel was dried and autoradiographed. Note that there is a fragment in D that is resistant to Mnl I digestion that is not present in the normal control. The fragment sizes in D are consistent with one of his alleles having a mutation at codon 119 that abolishes an Mnl I site.

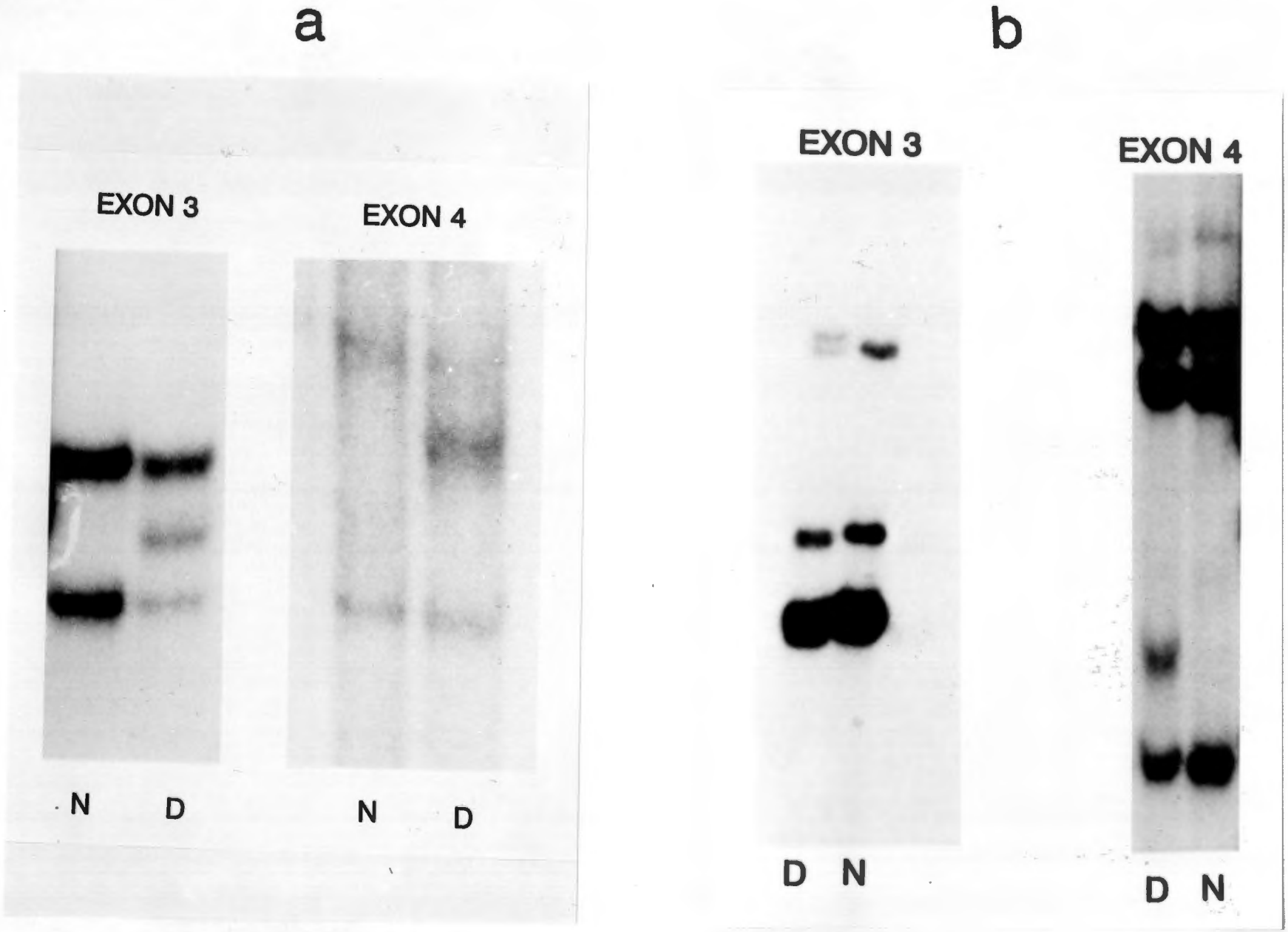


of the appropriate PCR product prepared from genomic DNA (Fig. 4.12). The resistance of part of the PCR product of D to normal Mn1 I digestion was evidence that this was a true mutation.

4.2.6 Population Analysis of Mutations in D

Twelve apparently unrelated Indian families with unequivocal FH (raised total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5 mmol/l plus xanthomata or thickened Achilles tendons) and 28 hypercholesterolemic patients without xanthomata were made available to us by the main lipid clinics in the provinces of the Cape, Transvaal and Natal. (There are virtually no Indians in the Orange Free State Province of South Africa). Since these mutations did not create or delete any restriction sites that were appropriate for population screening (Mnl I is very expensive), an SSCP diagnostic assay was established for these mutations. The SSCP assay that initially allowed us to detect the presence of these mutations (see Fig. 4.7) was based on PCR products of cDNA, not genomic DNA, and was rather crude. For the screening assay, PCR products were prepared using one pair of oligonucleotides that amplified exon 3 and another pair that amplified the 5' region of exon 4 from genomic DNA. PCR products from D gave different patterns to the PCR products from normal controls on the SCP gels (Fig. 4.13a and 4.13b). Note that the gel in Fig. 7 was a 6% acrylamide, 10% glycerol gel run at room temperature in 1 x TBE while that in Fig. 4.13a was an 8% acrylamide gel with no glycerol run at 4°C in

Fig. 4.13: SSCP analysis of the exon 3 and exon 4 mutations in D. Exon 3 and part of exon 4 of the LDL receptor were amplified by PCR from genomic DNA using oligonucleotides 5 and 3.1 and G31 and 4.3 respectively (see table 2A.1). The PCR products were radiolabelled by [α^{32}P]dCTP incorporation. PCR products from a normal subject and D were denatured in formamide and either electrophoresed on 8% polyacrylamide containing 0.5x TBE and no glycerol at 4°C at 8W (13a) or on a 50% Hydrolink gel containing 0.6x TBE and no glycerol at room temperature at 8W (13b). The conditions used in Fig.13a were used for most of the population screening.


a
EXON 3
EXON 4
N
D
N
D
b
EXON 3
EXON 4
D
N
D
N

½ x TBE. Fig. 4.13b shows SSCP results for these mutations on a 50% Hydrolink gel run in 0.6 x TBE. The conditions of Fig. 4.13a were used for the population screening. None of the 36 patients screened had either of the mutations detected in D (see Table 7.1).

4.3 DISCUSSION

Patient D has been characterised as a heteroallelic FH homozygote with point mutations at highly conserved amino acids in repeats 2 and 3 of the ligand-binding domain of the LDL receptor (Fig. 4.14). The mutation in repeat 3 could be particularly deleterious as it replaces the negatively charged glu with the positively charged lys in the triplet Ser-Asp-Glu near the carboxy terminus of the repeat. This triplet of negatively-charged amino acids is conserved in each of the ligand-binding repeats and is thought to be the ligand-binding site in each repeat. Ligand binding to the receptor is thought to involve an ionic interaction (Innerarity 1990). The substitution of the negatively charged Aspartyl residue with the aromatic Tyrosine residue in repeat 2 is also unlikely to be a neutral mutation.

These mutations were not detected in 72 alleles screened by SSCP (Table 7.1) and it is therefore unlikely that they are polymorphisms. Further evidence for the dysfunctional nature of these mutations is provided by analyses of mutations at homologous sites in different ligand-binding repeats. The Asp_{154}-His mutation commonly found in Sephardic Jews

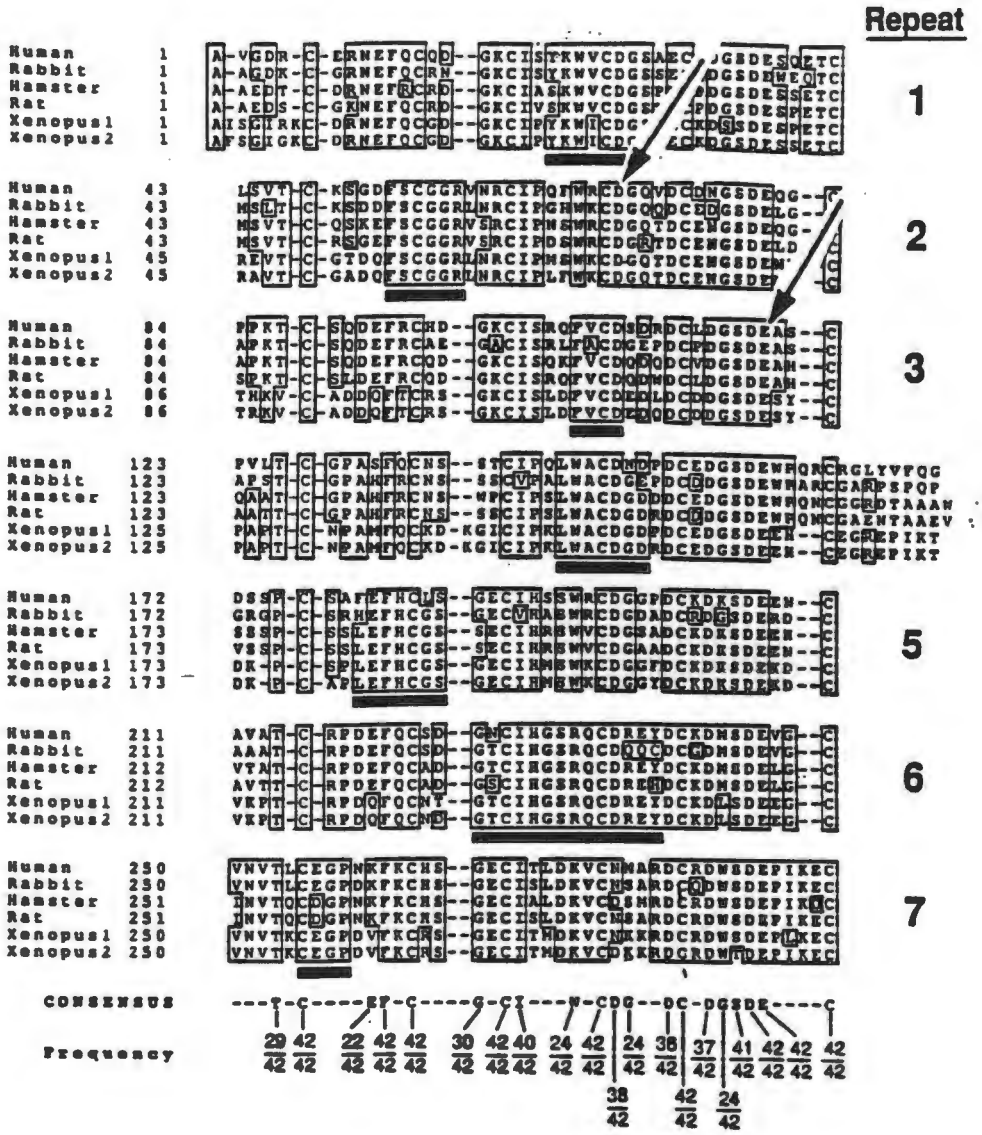


Fig. 4.14: A comparison of the amino acid sequences of the ligand-binding repeats in human, rabbit, hamster, rat and Xenopus showing the sites of the mutations in D (from Mehta et al. 1991a). The arrows show the sites of the Asp_{69}-Tyr and Glu_{119}-Lys mutations in repeats 2 and 3. The conserved amino acids within each repeat are blocked in and the consensus among the 7 repeats across the different species is noted at the bottom of the figure. The Asp that is mutated is present in 38 of the 42 positions while the Glu is present at the homologous position in each repeat in all the species examined here. Reproduced with permission of the authors and the American Society for Biochemistry and Molecular Biology.

(E. Leitersdorf, personal communication) is in the same site in repeat 4 as is D's Asp_{69}-Tyr mutation in repeat 2. The Sephardic mutation is clearly significant as the receptors are slowly processed, unstable and do not bind LDL but do recognise IgG-C7 (F. Graadt van Roggen, D.R. van der Westhuyzen, G.A. Coetzee and E. Leitersdorf, personal communication). A point mutation in repeat 2 that is thought to be functionally significant is the Trp_{66}-Gly French Canadian-4 mutation (Leitersdorf et al. 1990).

The French Canadian-3 mutation (Hobbs 1990), Glu_{207}-Lys (Repeat 5), (Leitersdorf 1990), is the exact parallel of D's Glu_{119}-Lys mutation in repeat 3. The French Canadian-3 mutation results in a slowly processed receptor (Leitersdorf et al. 1990) that does not bind ß-VLDL (Hobbs et al. 1990). The FH Lancashire mutation, Glu_{80}-Lys (Webb et al. 1992) occurs at the homologous site in repeat 2 to D's Glu_{119}-Lys mutation in repeat 3. This Glu_{80}-lys mutation results in a slowly processed (Type 2B) phenotype that forms a normal sized protein under reducing conditions, but an abnormally slowly migrating protein on non-reducing gels (Webb et al. 1992). The dysfunctional nature of these mutations at homologous sites in different repeats suggests that these sites are critical in maintaining the correct structure of the repeat and may therefore play the same role in different repeats.

Site-directed mutagenesis at these particular sites has not yet been performed, but the experiments of Russell et al. (1990) can be used to predict the effects of these mutations on ligand binding. Table 4.2 summarises the effects on LDL and ß-VLDL binding of the point mutations or the deletions in repeats 2 or 3 . It should be noted that the Asp_{118} that was mutagenised in repeat 3 is adjacent to the Glu_{119} that is mutated in D.

These mutagenesis experiments yield 3 important findings relevant to D's mutations.

1. None of the point mutations or the deletions of repeats 2 or 3 affected ß-VLDL binding to the LDL receptor.
2. The mutations in a given repeat had a similar effect on LDL binding whether a single amino acid was mutated or if the whole repeat was deleted. This result did not only hold true for LDL binding but also for ß-VLDL binding in parallel mutations from repeat 1 to repeat 7 in the ligand binding domain (Russell et al. 1990). This suggested that the individual binding repeats function in a modular manner. If a point mutation in a particular repeat disturbs binding then that repeat is functionally eliminated from the receptor but the other repeats can function undisturbed.
3. Mutations in repeat 2 caused a moderate reduction while mutations in repeat 3 result in severe impairment of LDL binding (Russell et al. 1990) (see Table 4.2).

Table 4.2: The effect of various mutations in repeats 2 and 3 in the ligand binding domain of the LDL receptor on LDL and ß-VLDL binding (from Russell et al. 1989).

Site-specific mutagenesis was used to create the following mutations in repeats 2 and 3 in the ligand-binding domain of the LDL receptor: either an entire repeat was deleted or Ile_{62} or Ile_{101} were changed to Asp or Asp_{79} or Asp_{118} were changed to Tyr. The point mutations were made at conserved sites. The values of specific binding of LDL and ß-VLDL to the cells containing plasmids expressing mutant DNA was expressed as % normal value (ligand bound by mutant cells/antibody bound by mutant cells ($ngx10^{-2}$/ng IgG bound) ÷ ligand bound by normal cells/antibody bound by normal cells ($ngx10^{-2}$/ng IgG bound) x 100).

Repeat	Deletion				Ile-Asp (residue 62 or 101)				Asp-Tyr (residue 79 or 118)			
	^{125}I-ligand binding (% control)				^{125}I-ligand binding (% control)				^{125}I-ligand binding (% control)			
	LDL		ß-VLDL		LDL		ß-VLDL		LDL		ß-VLDL	
	0.5 µg/ml	5 µg/ml	0.3 µg/ml	3 µg/ml	0.5 µg/ml	5 µg/ml	0.3 µg/ml	3 mg/ml	0.5 µg/ml	5 mg/ml	0.3 µg/ml	3 µg/ml
2	48	68	120	119	70	77	123	103	57	74	115	112
3	11	29	120	100	7	15	119	114	11	11	94	97

Thus, it is likely that neither of D's mutations will affect ß-VLDL binding. However, LDL binding to receptors with the Asp_{69}-Tyr mutation will probably be mildy reduced and LDL will probably be only very poorly recognised by receptors with the Glu_{119}-Lys mutation. Thus, the reduced LDL binding affinity in the face of normal ß-VLDL and IgG-C7 binding that was observed in D's fibroblasts was in accord with the results predicted by the mutagenesis experiments of Russell et al. (1990), especially if the codon 119 mutants comprise a significant proportion of the surface receptors in this patient.

The normal cholesterol levels in the obligate heterozygote mother of patient D is a stimulus for speculation that I cannot resist. Unfortunately, unless this family can be tracked down the following hypotheses are not likely to be testable. The one possibility is that D's mother carries a cholesterol-lowering gene that compensates for the effects of her mutant LDL-receptor allele (Hobbs et al. 1989). Another possibility is that the mutation in repeat 2 (Asp_{69}-Tyr) might be functionally mild like FH-Tonami-2, a deletion of repeats 1 and 2 that results in a 40% reduction in LDL affinity. Heterozygotes for FH-Tonami-2 have normal to only moderately raised (315 ± 80 µg/dl) total cholesterol levels. Four true homozygotes have been detected and are alive at 33, 48, 51 and 62! The FH-Tonami-2 heterozygotes also seem to live longer than "classical" heterozygotes (Kajinami, 1988).

The combination of such a mild mutation (repeat 2) with a more severe defect (repeat 3) predicted from the mutagenesis experiments discussed earlier, would probably result in a homozygote with typical features. A similar situation of unusually low cholesterol levels in the obligate heterozygote parents, especially the mother, of severely hypercholesterolemic FH homozygote monozygote twins, has been described (Vauy et al. 1991). This study suggested that the co-inheritance of mild to moderate defects of LDL-receptor function from both parents caused the FH homozygote phenotype in the twins.

The single-strand conformational polymorphism technique of detecting point mutations was used in two different ways in D. Initially it allowed us to detect the regions of the gene where the mutations were. In a sense this was fortunate, as this method does not detect all point mutations especially when the fragments are much longer than 200 b.p.. Since no cheap restriction sites were created or deleted by these two mutations (Mn1 I is very expensive), easy and reliable SSCP assays were established. Neither of the mutations were detected in 36 hyperlipidemic patients (including 8 patients with definite FH) that were tested (see Table 7.1). This result was not that surprising as the majority of Indians in South Africa came from many regions all over India and have only been resident in South Africa for about 3 generations. D is from a Hindu family. About 80% of South Africa's Indian population are Hindu whose marriage choices would have been

and are still constrained by the caste system. Therefore, any type of recent founder effect for this mutation in South Africa would be unlikely due to the large size of the founder population (150 000), the lack of time (3 generations) and the caste barriers that prevented the mutations from spreading throughout the population.

CHAPTER 5

IDENTIFICATION AND PROPERTIES OF THE PROLINE$_{664}$-LEUCINE MUTANT LDL RECEPTOR IN SOUTH AFRICANS OF INDIAN ORIGIN

5.1 INTRODUCTION

In the initial phases of these studies of familial hypercholesterolemia in South Africans of Indian origin, it was found that the LDL receptors from two homozygous patients, AV and AA, behaved very similarly. One of the LDL-receptor mutations that had been characterised in some depth at about the time this project was started was the FH Zambia mutation, so named because it was initially identified in MM, a Zambian of Indian origin (Soutar et al. 1989, Knight et al. 1989). This mutation results in a C to T transition in codon 664 that causes the substitution of proline by leucine. This single amino acid change occurs in the C repeat of EGF-precursor homology domain of the LDL receptor, distant from the repeats known to be essential for ligand binding. Interestingly, however, it was reported that this mutation markedly diminished the affinity of the receptor for LDL, and to a lesser extent ß-VLDL (Knight et al. 1989, Soutar et al. 1989).

We subsequently identified this same mutation in AA and AV (both homo-allelic homozygotes) and two other heterozygote subjects. The families of all four probands are unrelated and trace their origin to the vicinity of Surat in the Gujerat province of India. The availability of two subjects

homozygous for this mutation enabled us to re-examine its phenotype particularly with regard to ligand binding. When fibroblasts from AA and AV were studied, the mutant LDL receptors were found to have normal affinities for the ligands LDL and ß-VLDL. The disease FH in these subjects can be explained by low steady-state levels of receptor molecules that are functionally normal but exhibit accelerated turnover.

5.2 RESULTS

5.2.1 Identification of Pro_{664}-Leu Mutation

The CCG (proline) to CTG (leucine) mutation at codon 664 creates a Pst I restriction site in the LDL-receptor gene (Soutar et al. 1989). This mutation was detected by amplifying genomic DNA by PCR using primers flanking exons 13 and 14, and digesting the 496 bp product with Pst 1 (Fig. 5.1). The generation of a 381 bp and a 115 bp fragment indicated the presence of the mutant sequence CTG in exon 14 at codon 664. The possibility that two such fragments resulted from a mutation that created a Pst I site in exon 13 (i.e. at the 5'-end of the amplified fragment), rather than in exon 14, was ruled out: when genomic DNA was amplified using an upstream primer within rather than flanking exon 13 and the same downstream primer flanking exon 14, the Pst I digest of this product again released a 115 bp fragment. We have identified the $proline_{664}$-leucine mutation in FH patients from 4 unrelated families of Indian origin (Table 5.1). All the families are Muslims who originate from the

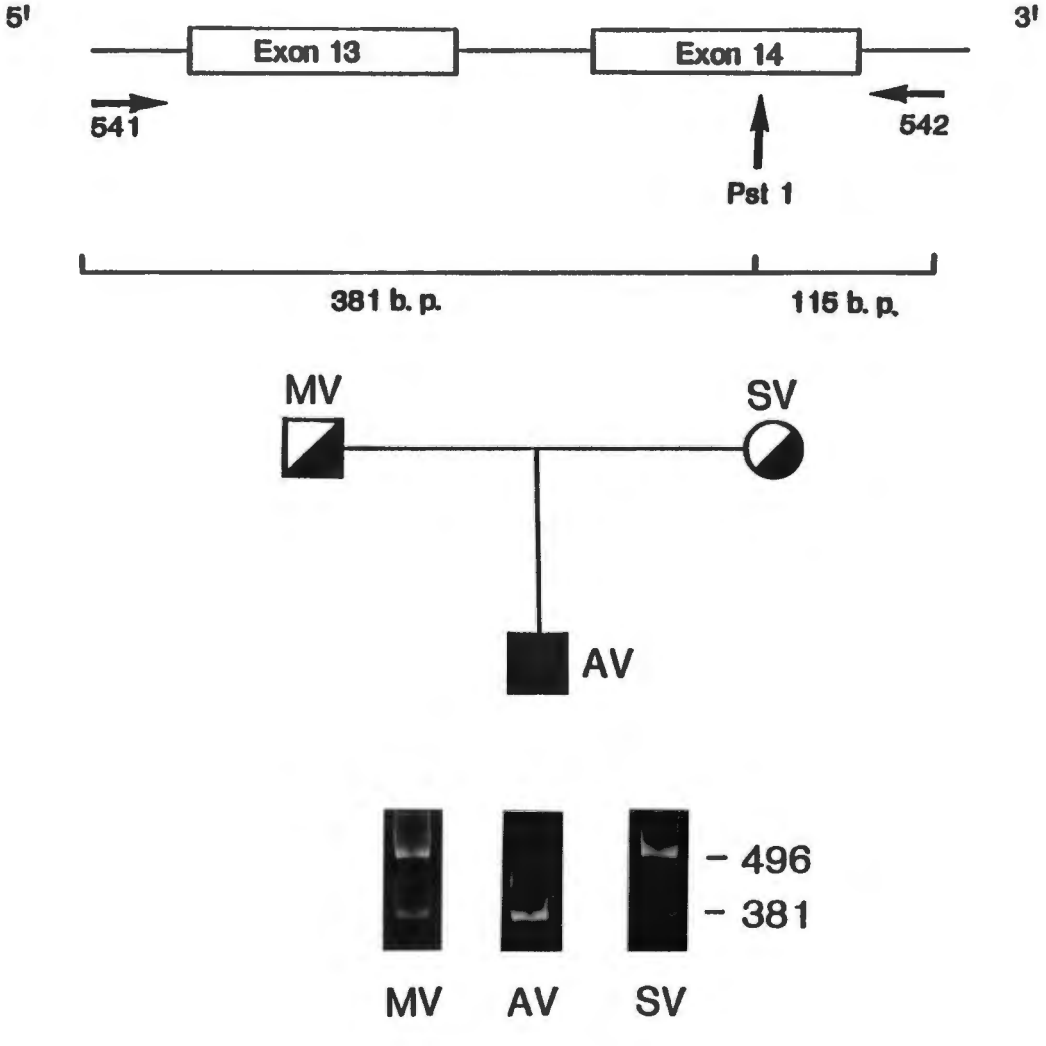


Fig. 5.1

(a) Diagram of the LDL-receptor gene showing the positions of oligonucleotides 541 and 542 and the Pst 1 site created by the Leu_{664} mutation.

(b) Polyacrylamide gel stained with ethidium bromide showing the Pst 1 digests of the 541-542 PCR products in the V family.

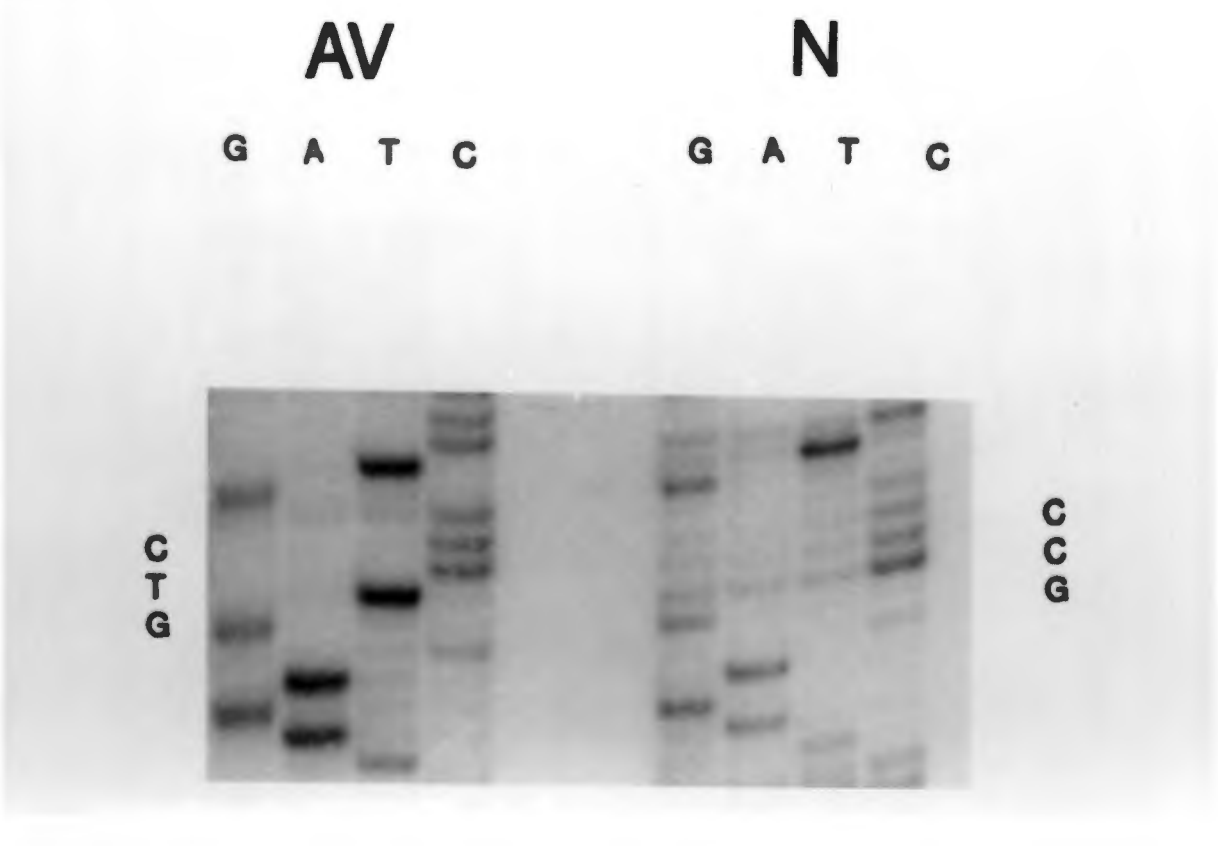


Fig. 5.2: DNA sequence of the Pro_{664}-Leu mutation in AV. DNA from AV and N cells was amplified using oligonucleotides 541 and 542. The PCR products were electrophoresed on 1% Agarose gels and the bands were eluted using Geneclean II. These PCR products were sequenced using kinased 542 and the fmol sequencing kit (Promega). This mutation was also confirmed in AA by direct sequencing (result not shown).

Table 5.1: Biochemical and clinical data for patients and affected relatives.

Individual	Sex	Age	TC	LDL-C	HDL-C	TG	FH664 Status	Sequelae
Family V								
AV (proband)	M	10	21.6	20.1	0.9	1.3	homo-zygote	Arcus Xanthomata (at age 3) Xanthelasmata
Mrs SV (mother)	F	42	7.6	5.8	1.4	0.6	hetero-zygote	Nil
Mr MV (father)	M	47	7.1	5.7	1.1	0.7	hetero-zygote	Arcus, Xanthomata
Family A								
AA (proband)	F	16	17.7	16.3	0.9	1.1	homo-zygote	Xanthomata (at age 5) Bruits
Mrs HA (mother)	F	43	8.0	6.1	1.1	1.8	hetero-zygote	-
Family MA								
FMA	F	47	8.3	6.4	1.7	0.5	hetero-zygote	Arcus Xanthelasmata I.H.D.
Family MT								
AMT	M	36	9.2	7.3	1.2	1.7	hetero-zygote	Arcus Xanthlasmata I.H.D.

TC = Total cholesterol, LDL-C = LDL-cholesterol, HDL-C = HDL-cholesterol, TG = triglyceride, all measured in mmol/l.

I.H.D. = ischaemic heart disease.

vicinity of Surat in the Gujerat province on the west coast of India. The unrelated homozygous probands AV and AA are offspring of different consanguinous marriages between first cousins. This mutation was confirmed in the homozygotes AV and AA by direct sequencing of the above PCR products (Fig. 5.2).

5.2.2 Haplotype of LDL-receptor Alleles in AV

More recently, the FH Zambia mutation has been identified in a Norwegian family on the same LDL-receptor haplotype as MM and, interestingly, in a number of English subjects in which the mutation was on a different LDL-receptor haplotype (King-Underwood et al. 1991). The five-enzyme haplotype of both LDL receptor genes in the FH patient, AV, was the same as that of MM (Ava II++, HincII--, Stu 1++. Taq 1--, Nco 1++) and distinct from the haplotype of the English subjects, suggesting that AV and MM inherited the same mutant LDL-receptor alleles. The haplotype of the British subjects differed from that of AV and MM at three restriction sites.

5.2.3 LDL receptor Synthesis, Processing and Turnover in AV's Fibroblasts

LDL receptor synthesis and processing was studied using [^{35}S]-methionine pulse-chase experiments in cultured fibroblasts. The precursor receptors in AV cells were abnormally slowly processed to mature forms with a half-life of conversion of about 2 hours (Figs. 5.3 and 5.4). Most of these precursor receptors were converted to the mature form

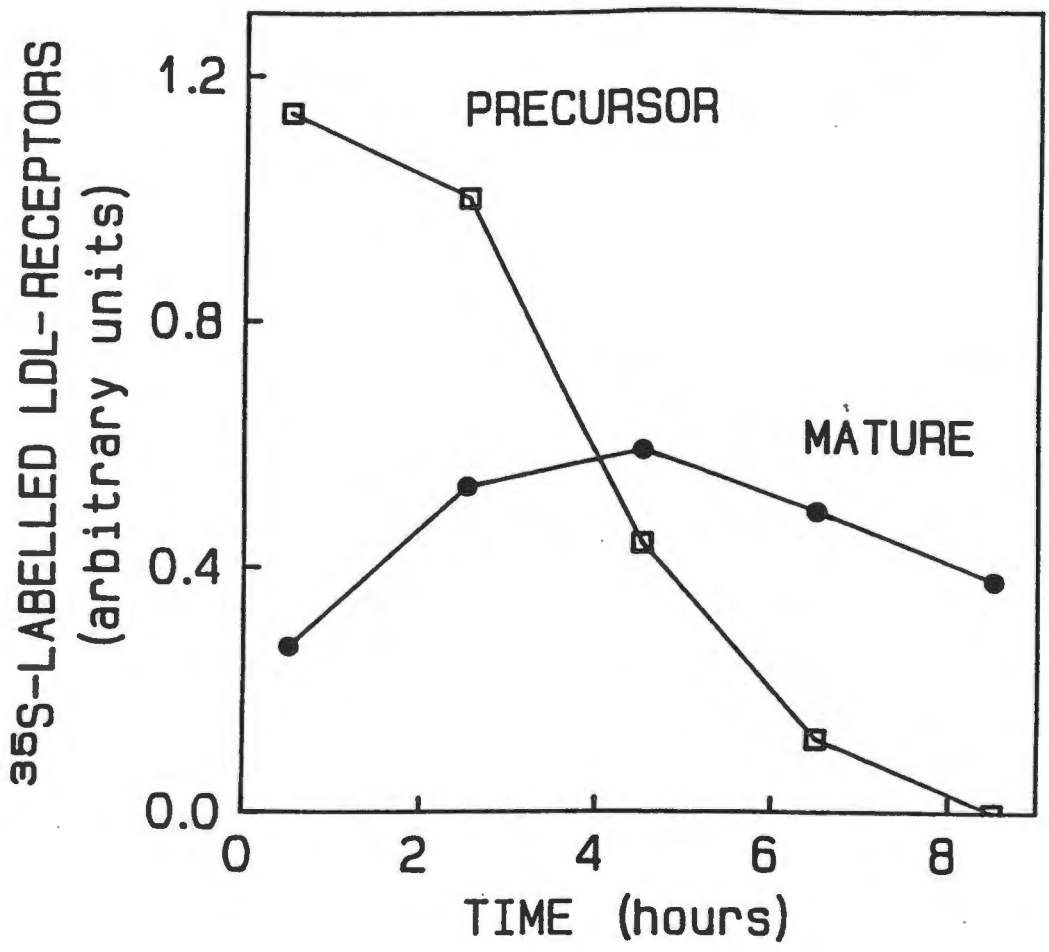


Fig. 5.3: Decay of radioactivity from pulse-labeled LDL-receptor protein.

Up-regulated fibroblasts from AV were pulse-labeled with [^{35}S]-methionine for 1.5 h and chased in DMEM/LPDS for the indicated times. The radioactivity in the 120 kDa precursor and 160 kDa mature receptors was quantified by densitometric scanning of autoradiographs of gels of immunoprecipitated LDL-receptors. All the values except those at 30 minutes are means of 2 experiments done in duplicate dishes. The values at 30 minutes are from 1 experiment only. In each experiment the intensity of the bands was expressed relative to the precursor band at 2.5 hours. In the same and other control experiments, normal receptor precursors were rapidly and completely converted to mature forms within 30 min of the chase period.

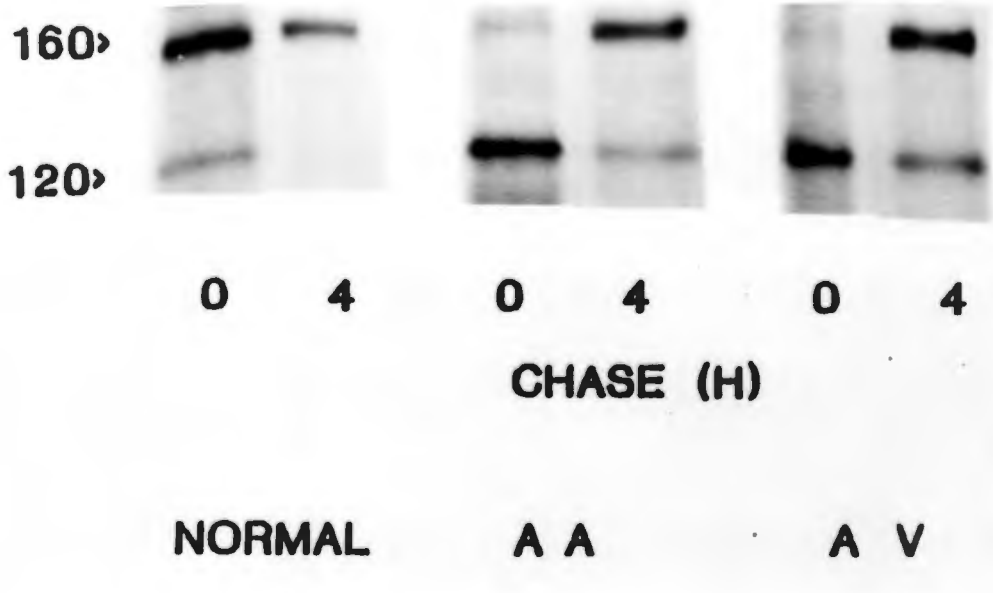


Fig. 5.4: Autoradiographs of immunoprecipitated [^{35}S]-labeled LDL-receptors from normal, AV and AA fibroblasts.
Up-regulated fibroblasts were pulse-labeled with [^{35}S]-methionine for 1 hour and chased in DMEM/LPDS for the indicated times. LDL-receptors were immunoprecipitated and visualised on autoradiographs as indicated in the Methods. The apparent molecular weights of the precursor (120 kDa) and mature (160 kDa) receptors are indicated.

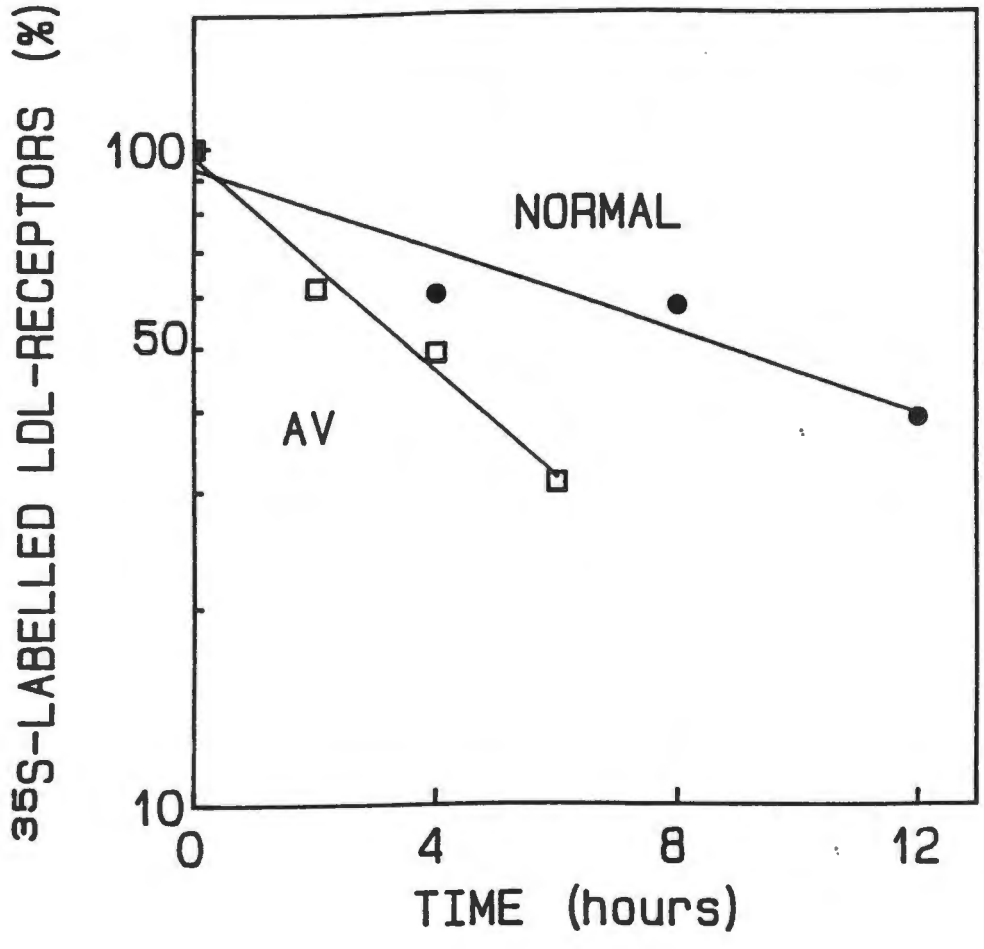


Fig. 5.5: Half-lives of mature LDL receptors from normal and AV fibroblasts

Normal and AV upregulated fibroblasts were pulse labeled with [^{35}S]-methionine for 40 minutes and 90 minutes respectively. The medium was then changed to DMEM/LPDS and the normal cells were chased for a ½ hour and the AV cells were chased for 6.5 hours before starting to measure the half-life of mature LDL receptors at time 0. LDL receptors were immunoprecipitated, run on 7% acrylamide and visualised on autoradiographs as indicated in the Methods. The points are the mean values from 2 experiments. Normal and AV fibroblasts were compared in each experiment in which each point was the mean of the values obtained in duplicate dishes. The half-lives of the mature LDL receptors from normal and AV fibroblasts were determined in the same 2 experiments.

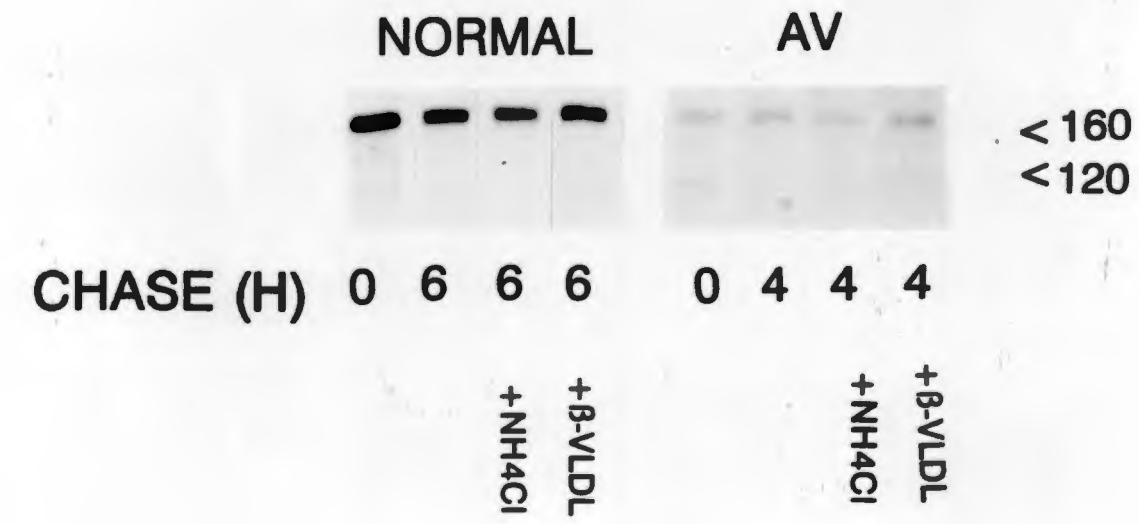


Fig. 5.6: Effect of ß-VLDL and NH_4Cl on turnover of normal and AV. Upregulated cells were pulsed with [^{35}S]-methionine for 1 hour. Normal cells were pre-chased for 2 hours and AV cells were pre-chased for 6 hours in DMEM/LPDS in order to convert most of the receptors to the mature form. The cells were then chased for the indicated times in DMEM/LPDS supplemented where indicated with either 10 mM NH_4Cl or 10 µg/ml ß-VLDL, before immunoprecipitation. The immunoprecipitates were electrophoresed on 7% SDS-PAGE and were visualised as described in the general methods.

(Fig. 5.4) although the possibility that some precursor degradation may have occurred could not be ruled out. By contrast, in the same experiments, normal receptor precursors (120 kDa) were rapidly and completely converted to the mature form (160 kDa) within 30 min of a chase period (data not shown). The retarded processing of the mutant receptors was presumably a consequence of a structural abnormality. We determined whether such an abnormality would affect the stability of the mature mutant receptors. The half-life of the AV's mature LDL receptors was about 3.5 hours, in comparison to a half-life of about 9 hours for normal receptors (Fig. 5.5).

Some mutations in the EGF-precursor homology domain result in impaired acid-dependent ligand-receptor dissociation (Davis et al. 1987a). Such mutant receptors are trapped in the cell in the presence of ligand and are subsequently rapidly degraded (Davis et al. 1987a). Since the degradation rate of AV's receptors was not abnormally enhanced when ß-VLDL (10 ug/ml) was added to the chase medium (Fig. 5.6), it was unlikely that this mutation resulted in impaired acid-dependent ligand-receptor dissociation. The inability of the lysosomotropic agent, NH_4Cl, to protect these mutant receptors from enhanced degradation suggested that the first steps in their degradation pathway were non-lysosomal (Fig. 5.6).

5.2.4 Ligand Binding and Metabolism in FH-664 Fibroblasts

AV and AA fibroblasts were able to sustain internalisation and degradation of ^{125}I-LDL at 37°C for 4 h at rates commensurate with their steady-state binding activity of about 25% of normal levels (Table 5.2). The internalization index for the mutant cells was normal, therefore indicating that the mutant cells internalised and degraded LDL normally and that their receptors recycled back to the surface appropriately. The rapid degradation of mutant receptors (see above) was therefore not further accelerated by the presence of ß-VLDL or LDL. Therefore, the FH Zambia mutation does not affect acid-dependent ligand-receptor dissociation. The ^{125}I-LDL concentrations that resulted in half-maximal ^{125}I-LDL degradation were similar in normal and mutant cells (Fig. 5.7). This suggested that the mutation does not affect the affinity of the receptor for LDL at 37°C. The maximum binding of AV cells at ligand saturation was 23% of normal.

The binding of ^{125}I-LDL and ^{125}I-IgG-C7 to AV cells was also studied at 4°C. The maximum number of surface receptors on the mutant fibroblasts that bind these ligands was about 20% of that in normal cells (Fig. 5.8). These binding curves indicated that the affinity of AV's receptors for both ligands was normal (Fig. 5.8).

Table 5.2: High-affinity binding, intracellular content and rates of degradation of ^{125}I-LDL in cultured fibroblasts

LDL metabolism at 37°C was done at 10 μg/ml ^{125}I-LDL as described in the Methods. Values are the means of 3 experiments each done in duplicate dishes. The internalisation index was calculated by dividing the sum of the ^{125}I-LDL degraded in 4 hours and the intracellular ^{125}I-LDL by the ^{125}I-LDL bound.

Cell strain	^{125}I-LDL			Internalisation
	Bound	Intracellular	Degraded	Index
	ng/mg cell protein			
Normal	198±55	1200±750	2990±1150	17-26
	% Normal			
AV	15±3	24±9	25±7	29-42
AA	25±9	27±9	29±8	20-27

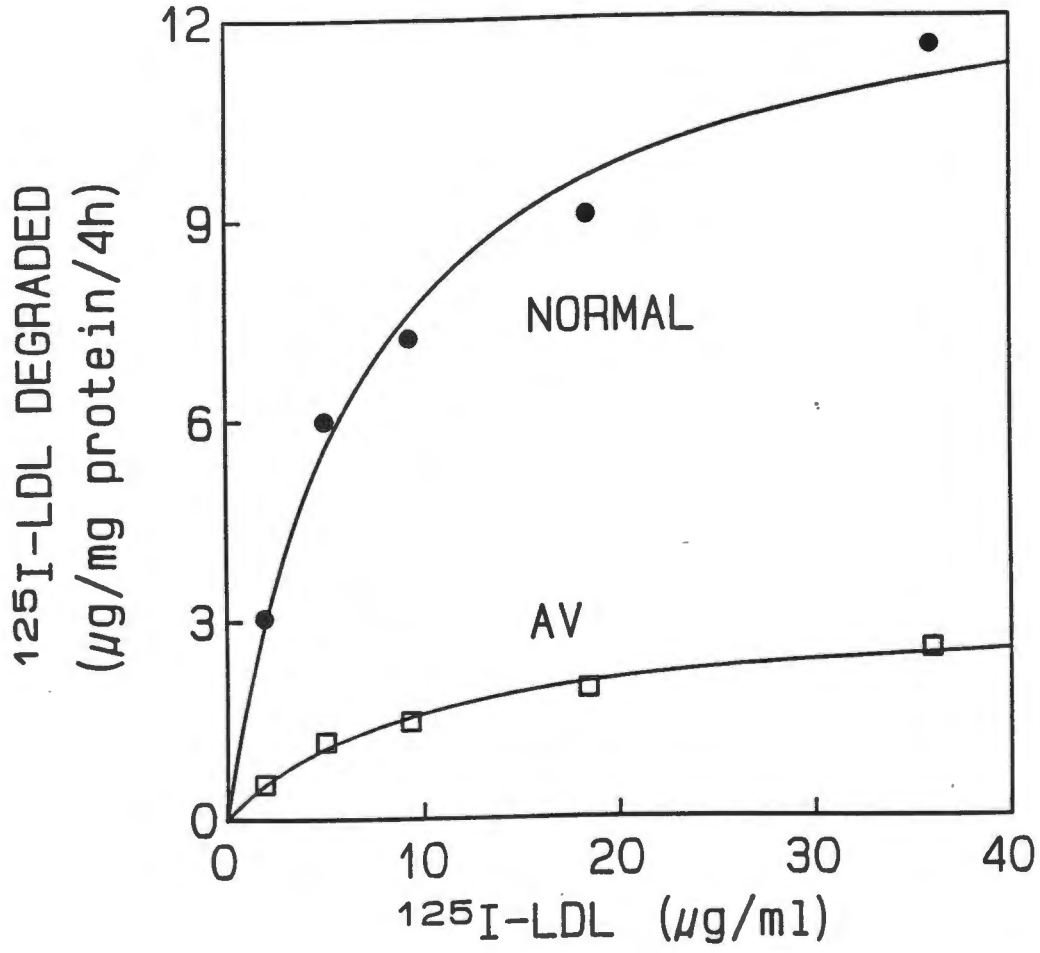


Fig. 5.7: ^{125}I-LDL degradation in normal and AV fibroblasts

Normal and AV upregulated fibroblasts were incubated with the indicated concentrations of ^{125}I-LDL for 4 hours at 37°C. The medium was collected and its content of non-iodide trichloroacetic acid-soluble radioactivity was determined and considered as the ^{125}I-LDL degraded in 4 hours (each point is the mean value of duplicate incubations). AV's fibroblasts degraded ^{125}I-LDL with a B_{max} of 23% normal. The ^{125}I-LDL concentration that resulted in half-maximal ^{125}I-LDL degradation was 5.1 μg/ml and 3.6 μg/ml in the mutant and the normal fibroblasts, respectively.

The K_d values for ^{125}I-LDL binding to normal and AV receptors were 1.8 ± 0.4 μg/ml and 1.8 ± 0.9 μg/ml, respectively. Cells from the other patient (AA) who was homoallelic for the FH Zambia mutation bound ^{125}I-LDL with an affinity similar to that of AV and normal fibroblasts (results not shown). These values were calculated by non-linear regression analysis of the total ligand binding values assuming one specific high-affinity binding site and a non-saturable component. Similar K_d values for normal and mutant cells were obtained when specific receptor binding was determined by two alternative approaches: non-specific binding was measured either in the presence of excess unlabelled ligand or in down-regulated cells and subtracted from total binding values. In these cases the calculated specific binding values were plotted and analyzed as single component curves (Fig. 5.9). The binding of an alternate lipoprotein ligand, ß-VLDL, was also examined. The ß-VLDL concentration required for half-maximal inhibition of ^{125}I-LDL binding at 4°C was similar in normal and AV cells, indicating that the affinity of the mutant receptor for this ligand was also normal (Fig. 5.10).

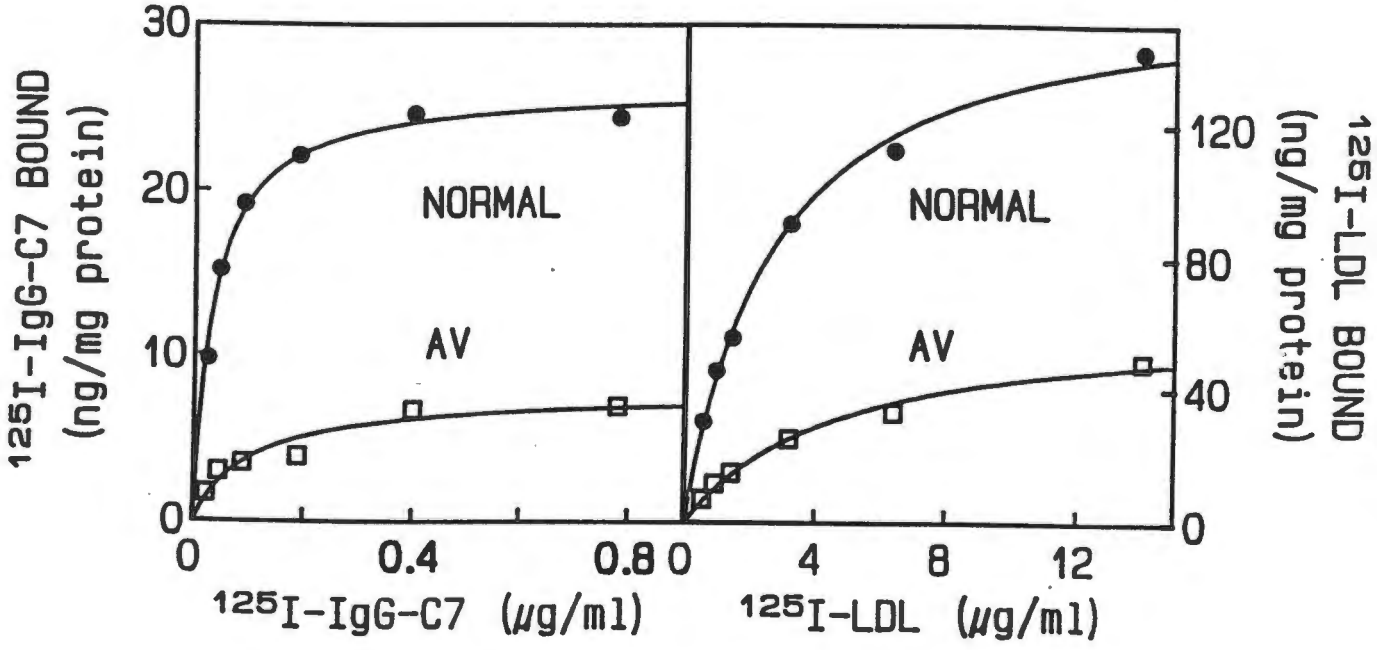


Fig. 5.8: 4°C binding of ^{125}I-LDL and ^{125}I-IgG-C7 to Normal and AV fibroblasts

Upregulated cells were incubated with varying concentrations of these ligands at 4°C for 2 hours and the total ^{125}I-ligand bound was determined as described in the Methods. Each value represents the average of duplicate incubations. AV fibroblasts bound IgG-C7 with a B_{max} of 20% of the normal fibroblasts and with a K_d of 0.05 μg/ml (Normal K_d = 0.04 μg/ml). AV fibroblasts bound ^{125}I-LDL with a B_{max} of 25% of the normal fibroblasts and with a Kd of 1.97 μg/ml (Normal K_d = 1.81 μg/ml). When these results were pooled with those from 2 other similar experiments, AV fibroblasts bound ^{125}I-LDL with a B_{max} of 20±9% of normal fibroblasts and with a K_d of 1.8±0.9 μg/ml as compared to 1.8±0.4 μg/ml in normal fibroblasts (mean ± standard deviation values).

Fig. 5.9: Comparison of specific binding of ^{125}I-LDL at 4°C derived by 3 different methods.

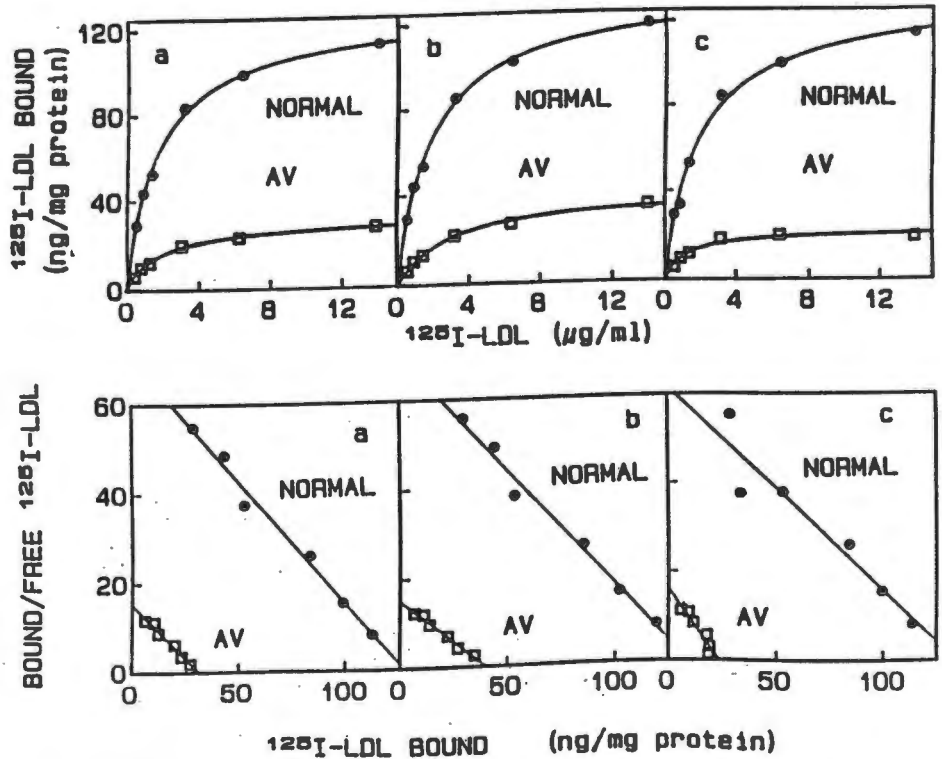
Fibroblasts, in a single experiment, were upregulated as described in the Methods and incubated at 4°C for 2 hours with varying concentrations of ^{125}I-LDL.

a. Total binding values were analysed using non-linear regression analysis assuming one specific binding site and a non-specific component (Enzfitter, Elsevier biosoft). Specific values are plotted. The calculated K_d values for the normal and mutant cells were 1.81 μg/ml and 1.97 μg/ml, respectively.

b. Non-specific binding was determined in the presence of excess unlabelled LDL (200 μg/ml). Non-specific binding was subtracted from total binding and the resultant specific binding values are plotted. The calculated K_d values of specific binding for normal and mutant cells were 1.99 μg/ml and 2.5 μg/ml, respectively.

c. Non-specific binding was measured in cells which had been down-regulated with 25-OH cholesterol for 48 h. Non-specific binding values were subtracted from total binding and the resultant specific binding values are plotted. The calculated K_d values of specific binding for normal and mutant cells were 1.84 μg/ml and 1.35 μg/ml, respectively.

The bottom panel in this figure shows the Scatchard plots of the corresponding binding data represented in the top panel.



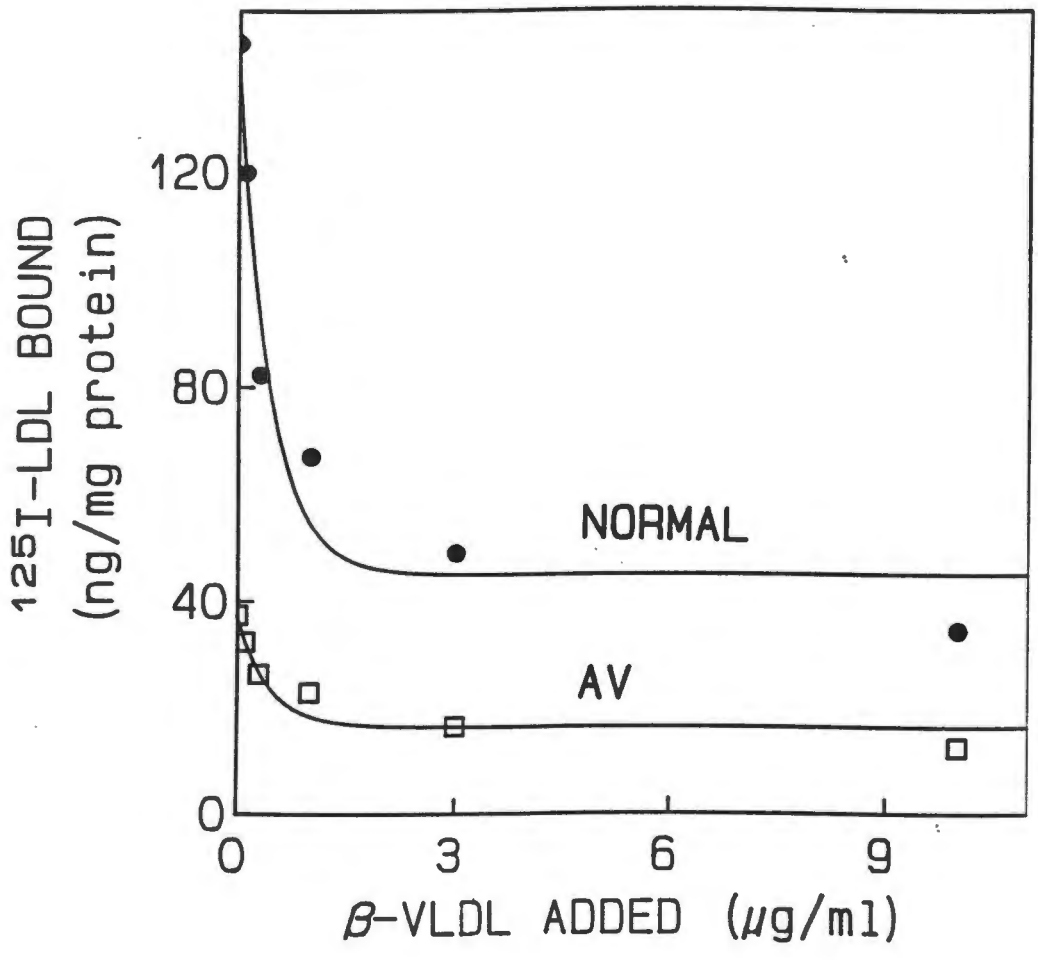


Fig. 5.10: ß-VLDL competition for ^{125}I-LDL bound to normal and AV fibroblasts.

AV and normal fibroblasts were incubated for 2 hours at 4°C with 2 μg/ml ^{125}I-LDL plus the indicated concentration of unlabelled ß-VLDL. Heparin-releasable ^{125}I-LDL binding was determined as in the Methods. Each value is the mean of duplicate incubations. Similar results were obtained in a separate experiment. The mean concentration of ß-VLDL required for half-maximal inhibition of ^{125}I-LDL binding to AV's fibroblasts calculated by non-linear regression (from two experiments) was 0.44 μg/ml as compared to 0.32 μg/ml in the normal fibroblasts.

5.3 DISCUSSION

The EGF-precursor homology domain of the LDL receptor is so-named because it encodes a sequence that is 33% identical to a part of the mouse epidermal growth factor-precursor gene (Südhof et al. 1985). Within this domain are 3 cysteine-rich 40 amino acid sequences, the first two (repeats A and B) that are contiguous and separated from the third (repeat C) by 280 amino acids (Südhofet al. 1985). These repeats are distinct from those in the ligand-binding domain and are found in a wide variety of extracellular proteins in humans and lower eukaryotes (Davis et al. 1987a).

The EGF precursor-like repeat C contains 6 cysteines that are thought to be disulphide-bonded to each other. It is likely that the first and third, the second and fourth, and the fifth and sixth cysteine within each of the repeats are covalently bonded, since this is the pattern that was found in other proteins with growth-factor repeats (Esser and Russell 1988). The three disulphide bonds in this 40 amino acid repeat probably result in a highly compact structure.

The abnormal function of many mutant LDL receptors can be predicted from the known structure/function correlations in the normal receptor (Hobbs et al. 1990).

Mutations in the EGF-precursor like domain seem to affect four distinct functions of the LDL receptor. Receptor transport from the ER to the Golgi is particularly sensitive

to major conformational changes. The most severe transport-defective mutations (Class 2A) produce receptors that are completely blocked from reaching the Golgi (Hobbs et al.1990). All five of the Class 2A mutations reviewed by Hobbs et al. (1990) occur in the EGF-precursor homology domain. These authors suggested that this domain probably has a highly compact structure that is easily disturbed by single point mutations. This may explain why this domain has been so highly conserved in evolution (Mehta et al. 1991).

Since receptor stability is likely to be at least partly determined by protein 3° structure, it is not surprising that mutations in the EGF-precursor homology domain affect LDL receptor turnover. At least two different mutations in this region, FH Cape Town-2 (van der Westhuyzen et al. 1991), and FH-Afrikaner-2 (Leitersdorf et al. 1989), are inherently unstable. The Asp_{412}-His mutation (Miyake et al. 1992) in exon 9 was processed normally, however no detectable ^{125}I-LDL or ^{125}I-C7 binding was detected on the surface of this subject's fibroblasts. It is likely that this mutation results in mature receptors that are very rapidly degraded and thus the pool size of surface receptors is very small. It is interesting to speculate about the possibility that the predominant effect of mutations in the EGF-precursor domain might be to affect the stability of mature receptors. If one had mutant receptors that were so unstable that the half-life of the mature forms was much shorter than the half-time of the rate of conversion of precursor to mature receptors

(assuming that conversion did occur, even if more slowly than normal), then one would probably not be able to detect mature receptors being formed in pulse-chase experiments. Such a mutation would be classed as type 2A, a complete block in transport! There is one way to possibly determine whether type 2A mutations are really the result of complete block in transport or a consequence of hyper-instability of mature LDL receptors. The degradation of normal receptors is inhibited by cycloheximide, and therefore is probably mediated by a short-lived protein(s) (Casciola et al. 1988) and is not affected by lysosomotropic agents (Casciola 1989). The degradation of certain mutant receptors might be lysosomal, however. If one re-examined all of the class 2A mutations by pulsing their cells for enough time to label a significant precursor receptor pool and then added cycloheximide and/or lysosomotropic agents, one might find that the extremely unstable putative mature forms would be protected from degradation and thus become detectable. Unfortunately, a negative result in such an experiment would not be informative.

One of the crucial features of the LDL receptor is its ability to separate from internalised ligand as a result of the decreased pH in the endosomes. The freed receptor can thus recycle to the cell surface. Deletion of repeats A and B or the entire EGF-precursor domain seem to affect this acid-dependent receptor-ligand dissociation in both natural (Miyake et al. 1989) and artificial mutants (Davis et al.

1987a). This deficiency results in rapid disappearance of mutant receptors from the cell surface in the presence of ligand, with subsequent intracellular trapping and degradation of receptors.

The EGF-precursor homology domain also plays a role in ligand binding. A mutant receptor that had this entire domain deleted bound both ß-VLDL and LDL on nitrocellulose blots but in intact cells only bound with high affinity to ß-VLDL, not LDL (Davis et al.1987a). It was suggested that the EGF-precursor domain positions the ligand-binding domain in a conformation that allows it to bind LDL on the cell surface. A similar reduction of LDL binding in the face of normal ß-VLDL binding was observed in naturally occurring mutants where either the entire EGF-precursor homology domain (Miyake et al. 1989) or just repeats A and B (van der Westhuyzen et al. 1991) were deleted. Artificial mutants, where either repeat A alone or both repeats A and B are deleted, demonstrate the same apo B-100-binding deficiency (Esser et al. 1989). However, deletion of repeat B alone does not affect LDL or ß-VLDL binding to the LDL receptor (Esser 1989). These results suggest that repeat A is the critical region of the EGF- precursor homology domain that determines LDL binding. The findings of Soutar and Knight on the MM mutation in repeat C were therefore of considerable interest (Soutar et al. 1989, Knight et al. 1989).

The proline at amino acid 664 is in a highly conserved region between cysteine 3 and cysteine 4 (Mehta et al. 1991) in repeat C. The substitution of leucine for proline at this site presumably results in aberrant folding of the mutant protein since proline causes fixed kinks in polypeptide chains and therefore probably plays an important role in determining conformation. This, in turn, could account for the slow processing and instability of this receptor, features common to other mutations in this domain (van der Westhuyzen et al. 1991, Leitersdorf et al. 1989). However, the report that this point mutation, more than 350 amino acids away from the ligand binding domain, affected the affinity of ligand binding, was unexpected (Soutar et al. 1989, Knight et al. 1989). It suggested a new role for repeat C either in the maintenance of the correct conformation of the binding domain or in controlling appropriate interactions between LDL-receptor molecules. The identification of 2 patients who were homozygous for this mutation allowed us to re-examine its phenotype.

Our results differ in one fundamental respect from those previously reported: Knight and coworkers reported that MM's receptors exhibited a decreased affinity for the ligands ß-VLDL (Soutar et al. 1989) and LDL (Soutar et al. 1989, Knight et al. 1989). The decreased affinity for LDL was apparent both at 37°C and particularly at 4°C, where the mutant receptor's Kd was 5 times the value obtained for normal receptors. Our affinity measurements indicate that the

mutant receptors have a normal affinity for IgG-C7, LDL and ß-VLDL at 4°C. The ^{125}I-LDL concentration that resulted in half-maximal ^{125}I-LDL degradation at 37°C was similar in normal and in mutant fibroblasts. This suggested that the affinity of these receptors for LDL at 37°C was also normal. The reason for the differences between our results and those previously reported is not clear.

The low number of surface receptors in AV's fibroblasts is at least partially due to the increased rate of receptor turnover. The enhanced degradation rate, also reported by Knight and coworkers (1989), was observed in the absence of lipoprotein ligand. The abnormally fast degradation rate of the mutant receptors was not further enhanced by the presence of LDL or ß-VLDL, since there was no gross depletion of mutant receptors after 4 hrs of incubation of cells with either ligand at 37°C. Therefore an impairment of acid-dependent ligand-receptor dissociation and the consequent trapping of receptors in endosomes does not explain the accelerated receptor degradation.

The absence of any inhibition of the degradation of these mutant receptors by the lysosomotropic agent, NH_4Cl, suggested that these receptors are initially degraded in a non-lysosomal compartment, like normal receptors (Casciola et al. 1989).

The diagnosis of this mutation in AV and AA relied on the creation of a Pst 1 restriction site and direct sequencing of exon 14. It is unlikely, but possible that another LDL-receptor mutation might co-exist on the same allele(s) as the FH Zambia mutation in our patients. We have established that our subject AV has the same haplotype as MM. It is unlikely that a second mutation could correct for a defect in binding caused by an initial mutation at amino acid 664.

The pro_{664}-leu mutation was originally identified in MM, a Zambian who originated from Gujerat on the West Coast of India. Subsequently this mutation was identified in another family of the same racial and geographical origin (King-Underwood et al. 1991). We have identified this mutation in four families. They are all of Gujerati Muslim origin from the vicinity of Surat. The frequent identification of this mutation in Indian emigrants of Gujerati/Surat origin suggests that this mutation might be very common in this area of India.

Muslim Gujeratis, until very recently, only married descendants from their own village or locality in India. Therefore this group was genetically isolated both in India and abroad. It is thus unlikely that this mutation would have been uniformly distributed among all Indians in South Africa of which the majority are Hindus. However, since Muslim Gujeratis comprise less than 1/5 of South Africa's 800 000 - 1 000 000 Indians (Lazarus 1976), the finding of 4

unrelated kindreds with this mutation suggests that this mutation is indeed very common in this group.

CHAPTER 6

FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100 (FDB) IN SOUTH AFRICA

6.1 INTRODUCTION

Familial Defective Apolipoprotein B-100 (Arg_{3500}-Gln) and LDL-receptor mutations are the known causes of monogenic hypercholesterolemia and are often clinically indistinguishable (Rauh et al. 1982, Tybjaerg-Hansen et al. 1990; see 1.8 for review). The Afrikaner, Jewish and Indian populations in South Africa each have a particularly high incidence of monogenic hypercholesterolemia (MH) (Seftel et al. 1980; Seftel et al. 1989; Seftel H and Asvat M, personal communication). Although major founder LDL-receptor mutations account for most of this condition in Afrikaners (Leitersdorf et al. 1989, Kotze et al. 1989) and South African Jews (Meiner et al. 1989), these mutations did not account for all of the monogenic hypercholesterolemia in the lipid clinics (Graadt van Roggen et al. 1991) and the question remained whether FDB was present in these populations.

The so-called Coloured population in South Africa is of mixed indigenous African, Indonesian and North European ancestry. Due to the significant input of European genes into the Coloureds' gene pool, as evidenced by the presence of FH Afrikaner-1 and FH Afrikaner-2 LDL-receptor mutations (M Kotze, F Graadt van Roggen, personal communication), the possibility existed that FDB might be found in this group.

Most of the Indian patients who were referred to us with the diagnosis of FH were negative for the LDL-receptor mutations that we had identified in this population. Therefore, we investigated this group for the presence of FDB, as most studies have only looked for this disorder in European/North American Caucasians.

Hypercholesterolemic patients with or without thickened Achilles tendons or xanthomata were screened for FDB. Initially these patients were analysed for the relevant common/founder LDL-receptor mutations. If the frequencies of the non-founder LDL-receptor mutations (Goldstein and Brown 1989) and FDB (Innerarity et al. 1990) were both 1/500, as is the case in Europe and North America, then one would expect equal numbers of LDL-receptor and FDB mutations in the patients with xanthomata or thickened Achilles tendons who did not have the founder mutations. This would of course only be the case if the two conditions were clinically identical.

Although no patients with FDB were identified in the above populations, two families (M and W) each of mixed Afrikaner and British ancestry were identified with this mutation. The M family that has FDB was initially screened along with the Jewish group as the proband (non-Jewish) had a Jewish husband/surname. The W family is unique to our knowledge as the pedigree contains both the FH Afrikaner-1 and FH Afrikaner-2 mutations and FDB in different combinations.

There are subjects with each of the individual gene defects but there are also 4 individuals that are mixed heterozygotes for FH Afrikaner-1 and FDB and one "complex" heterozygote that has both FH Afrikaner-1 and FH Afrikaner-2 LDL-receptor defects and FDB! This large pedigree allows the clinical comparison of the various genotypes and their combinations.

6.2 RESULTS

6.2.1 FDB in hypercholesterolemic Afrikaners, "Coloureds", Indians and Jews in South Africa

The results of our initial screening of the various South African populations for FDB are shown in Table 6.1. Patients were placed in 6 categories. Subjects that had total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5.2 mmol/l were called definite monogenic hypercholesterolemics (MH) if they had evidence of xanthomata or thickened Achilles tendons. Patients with similarly elevated total- and LDL-cholesterol levels and triglyceride concentrations <2.3 mmol/l with no xanthomata or thickened Achilles tendons were considered to have possible MH. The patients from the Tygerberg, Groote Schuur, Johannesburg General and R.K. Khan Hospital clinics were assessed by physicians for the presence of xanthomata and thickened Achilles tendons, features that are pathognomonic for heterozygous MH in the context of the appropriate lipid profile. The Afrikaners from Potchefstroom were unfortunately not assessed for these features and are therefore listed separately as there might be patients in the

possible MH group that would qualify for the definite MH group.

Patients with total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5.2 mmol/l and triglyceride concentrations of 2.3-4.5 mmol/l were called type IIb. They could also have LDL receptor mutations or FDB. If patients with similarly elevated cholesterol levels had triglyceride levels > 4.5, that make it impossible to determine LDL levels using the Friedewald equation (Thompson 1989), they were called mixed hyperlipidemics.

Mildly hypercholesterolemic subjects (total cholesterol > 6 mmol/l) were also analysed for FDB as some of the patients that were initially described with this condition manifested a milder disease than that caused by LDL-receptor mutations (Innerarity et al. 1990).

The patients in the Afrikaner and Jewish groups were all pre-screened for the presence of relevant LDL-receptor founder mutations (FH Afrikaner-1, -2 and -3 and FH Piscataway). Coloured patients were tested for the Afrikaner founder mutations. Patients who tested positive for these mutations were nevertheless screened for FDB but were placed in a separate category aside from those who were negative. Some of the patients in the Indian population were screened for the FH Zambia mutation and the two mutations we identified in D. The number of Indian patients in each category that were

Table 6.1: Summary of Patient Groups screened for the FDB Mutation [1]

	LDL-Receptor [2] mutation confirmed	Definite "MH" [3]	Possible "MH" [4]	Type IIb[5] hyper-lipidemia	Mixed [6] hyper-lipidemia	Mild [7] hyper-lipidemia
AFRIKANERS						
Clinically assessed	9	7	23	11	3	26
Potchefstroom group[8]			10	5		15
TOTAL AFRIKANERS	9	7	33	16	3	41
COLOUREDS	2	8	8	1	1	1
INDIANS		7(6)[9]	13(9)	8(8)	4(3)	6(3)
JEWS	3	2	3			3

1 All patients in this table were negative for FDB. Two patients each of mixed British and Afrikaner ancestry were identified with FDB and are described in the text. All patients were unrelated.

2 All Afrikaner and Coloured patients were screened for the FH-Afrikaner-1, -2 and -3 mutations. All Jewish patients were screened for the FH-Piscataway mutation. Patients who were positive were placed in this group and were screened for FDB but were considered aside from the other subjects.

3 Definite Monogenic Hypercholesterolemia "MH": Total cholesterol >7.5 mmol/l or LDL cholesterol > 5.2 mmol/l with presence of xanthomata or thickened Achilles tendon in patient or in 1st degree relative. Only the patients in this group had xanthomata or thickened Achilles tendons in themselves or 1st degree relatives.

4 Possible "MH": Total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5.2 mmol/l but Triglycerides < 2.3 mmol/l.

5 Type IIb hyperlipidemia: Total cholesterol > 7.5 mmol/l or LDL cholesterol > 5.2 mmol/l but triglycerides > 2.3 mmol/l and < 4.5 mmol/l.

6 Mixed hyperlipidemia: Total cholesterol > 7.5 mmol/l and Triglycerides > 4.5 mmol/l.

7 Mild hyperlipidemia: Total cholesterol > 6 mmol/l.

8 The Potchefstroom patients were the only group that were not clinically assessed therefore none of these patients could be placed in the definite "MH" group.

9 Most of the Indian patients were screened for the FH-Zambia and the 2 mutations in "D":. The number of patients that were screened for these mutations are indicated in brackets, while the total number of patients in each group that were screened for FDB are shown outside brackets.

screened for all three of these mutations (in addition to FDB) are in brackets.

The rationale for screening for these LDL-receptor mutations was to eliminate the founder mutations that resulted in the increased FH frequency in these groups from our analysis. The remaining sporadic LDL-receptor mutations presumably exist at a frequency of 1/500 (Goldstein and Brown 1989). Thus the frequency of FDB in the groups with definite MH would allow some estimate of the population frequency as the definite MH groups probably contain only LDL-receptor mutations and FDB.

Although FDB was not detected in any of the true Afrikaners, Jews, Indians and so-called "Coloureds" listed in Table 6.1, this mutation was identified in two families each of mixed Afrikaner and British ancestry.

6.2.2 THE M FAMILY

The genogram of the M family is shown in Fig. 6.1. The proband, her child and grandchild are heterozygous for FDB. The proband's father died from an M.I. at 42 and premature coronary artery disease seems to have affected at least one of his siblings and nephews. The absence of heart disease in the proband's mother and siblings suggests that the mutant gene came from the father's side. Unfortunately, this

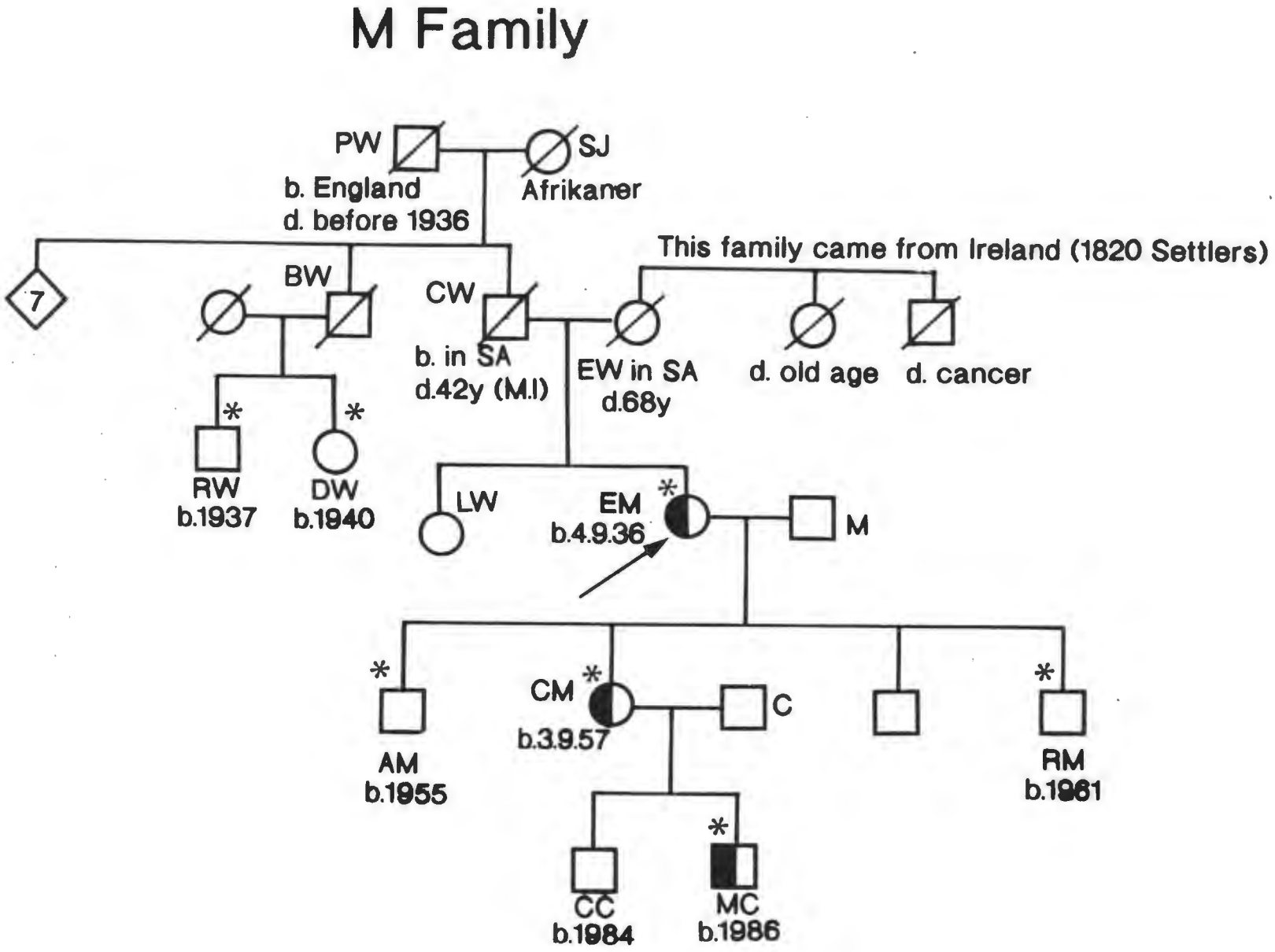


Fig. 6.1: Family tree of the M family.
Asterixes indicate patients who were screened for FDB.

Table 6.2: The M Family; Clinical and Biochemical details

Initials	FDB	SEX	Age of Sampling [1]	Arcus	Xanthelasma	Xanthoma	CHD	TC[2]	TG	HDL	LDL	Apo E Genotype [3]
EM	+	F	49	Y	N	N	N	8.02	1.53	0.96	6.4	3/4
CC	+	F	34	N	N	N	N	6.74	0.93	0.91	5.4	4/4
MC	+	M	6	N	N	N	N	5.06	0.69	0.80	3.9	2/4
AM	-	M	36	N	N	N	N	5.62	0.75	0.96	4.3	ND[4]
RM	-	M	31	N	N	N	N	5.82	1.91	1.10	3.8	ND
RW	-	M	55	N	N	N	Yes	5.40	1.17	0.88	3.9	ND
DS	-	F	52	N	N	N	N	6.38	2.07	1.01	4.4	ND

1 No patients were on lipid-lowering drugs at the time of sampling.
2 TC (total cholesterol), TG (triglyceride), HDL (high density lipoprotein) and LDL (low density lipoprotein) concentrations are all expressed in mmol/l.
3 Apo E genotypes were determined by the Hha I system (Hixson 1990)
4 Not done.

analysis does not help elucidate the origin of FDB in this family as the proband's father had British and Afrikaner parents.

A comparison of lipid levels in the unaffected and FDB subjects from this family (Table 6.2) suggests that the LDL-cholesterol levels in the affected individuals are higher than those in the normals. Each of the FDB subjects has a type IIa lipid profile. The only peripheral stigmata of hyperlipidemia were noted in the proband EM, who had a mild arcus cornealis that regressed subsequent to treatment with simvastatin. Her response to simvastatin treatment (up to 40 mg/day) was compatible with that reported in the literature (Maher et al. 1991). Her total cholesterol levels dropped from 8.02 mmol/l to 5.06-5.59 mmol/l and her LDL-cholesterol concentration dropped from 6.4 mmol/l to 3.6-4.1 mmol/l.

6.2.3 THE W FAMILY

The proband (CW) in the W family (Fig. 6.2) was initially considered to be an FH heterozygote . Her DNA was analysed for the FH Afrikaner-1 LDL-receptor mutation and for FDB since she was referred to us with high total cholesterol levels (15-18 mmol/l) and had an Afrikaner father. When both of these mutations were identified in CW, her very large family was recruited for further investigation (Table 6.3). Each member of the W family that was recruited was screened for FH-Afrikaner-1 and FDB and most of the hyperlipidemic subjects were positive for one or both mutations.

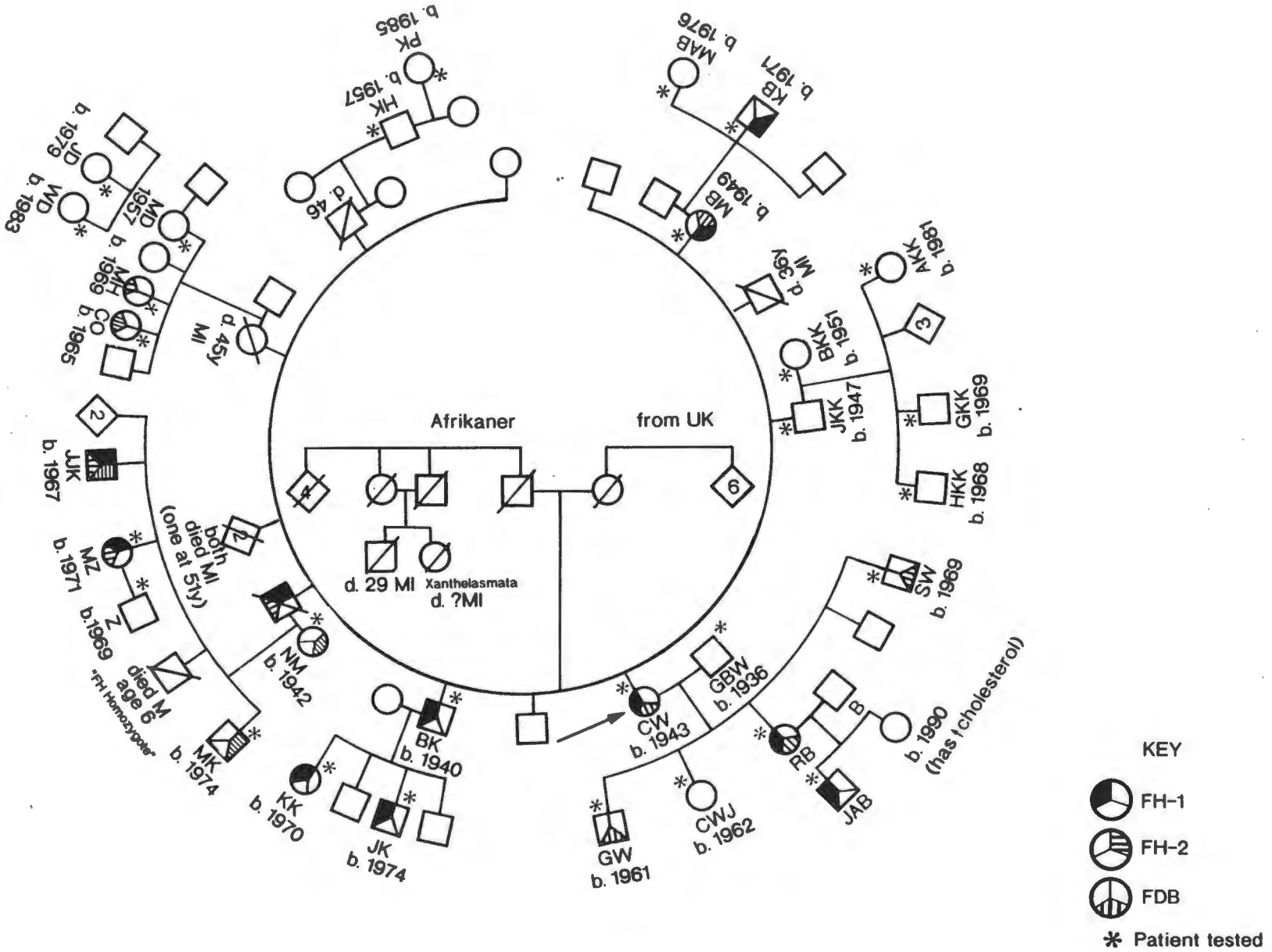


Fig. 6.2: Family tree of the W family. Asterixes indicate patients who were tested for the relevant mutations.

Table 6.3: Clinical Details of W Family

Initials	Sex	FDB	FH-1	FH-2	FH-3	Age at sampling	Corneal Arcus	Xanthelasma	Xanthomata Achilles	Xanthomata Other	CHD	TC[p]	TG	HDL	LDL	ApoE[q]
NO MUTATIONS DETECTED																
GBW	M	-	-	-	-	56	N	N	N	N	N	7.29	1.36	1.09	5.6	ND
CWJ	F	-	-	-	-	30	N	N	N	N	N	6.36	0.93	1.36	4.6	ND
HK	M	-	-	-	-	34	N	N	N	N	N	5.91	2.55	0.88	3.9	ND
PK	F	-	-	-	-	7	N	N	N	N	N	4.29	0.56	1.53	2.5	ND
MD	F	-	-	-	-	35	N	N	N	N	N	7.3	0.69	1.34	5.6	ND
JD	F	-	-	-	-	12	N	N	N	N	N	5.65	0.77	1.63	3.7	ND
WD	F	-	-	-	-	8	N	N	N	N	N	5.91	0.48	1.66	4.0	ND
PZ	M	-	-	-	-	23	N	N	N	N	N	7.05	2.24	0.99	5	ND
JKK	M	-	-	-	-	45	N	N	N	N	N	5.88	1.24	1.5	3.8	ND
BKK	F	-	-	-	-	41	N	N	N	N	N	4.81	3.2	0.73	2.6	ND
HKK	M	-	-	-	-	24	N	N	N	N	N	4.8	5.0	0.73	-	ND
GKK	M	-	-	-	-	23	N	N	N	N	N	4.51	1.05	1.05	2.97	ND
AKK	F	-	-	-	-	10	N	N	N	N	N	3.69	0.8	1.01	2.3	ND
MAB	F	-	-	-	-	16	N	N	N	N	N	5.66	0.94	1.22	4.0	ND
						28±14						[ca] 5.7 ±1.1	1.0 ±0.4	1.2 ±0.3	3.9[hj] ±1.2[ik]	
FH-AFRIKANER-1 (FH-1)																
JAB	M	-	+	-	-	5	N	N	N	N	N	7.18	0.65	1.39	5.5	3/3
BK	M	-	+	-	-	51	N	N	N	N	N	9.89	1.27	1.56	7.7	3/3
KK	F	-	+	-	-	21	N	N	N	N	N	10.78	1.01	1.88	8.4	3/3
JK	M	-	+	-	-	17	N	N	N	N	N	7.85	1.47	1.18	6	3/3
KB	M	-	+	-	-	20	N	N	N	N	N	7.96	0.96	0.82	6.7	2/3
						23±17						[a] 8.7 [e] ±1.5	1.1 ±0.3	1.4 ±0.4	6.9 [h] ±1.2[l]	
FH -AFRIKANER-2 (FH-2)																
NM	F	-	-	+	-	50	N	N	N	N	N	13.12	3.65	1.06	10	3/3
MK	M	-	-	+	-	23	N	N	N	N	N	8.73	1.36	1.23	6.9	3/3
FH-AFRIKANER-1 + FH-AFRIKANER-2 COMBINED AVERAGES						26±18						[d] 9.4 [f] ±2.1	1.5 ±1.0	1.3 ±0.3	7.3 [k, m] ±1.5	
FDB																
GW	M	+	-	-	-	31	N	N	N	N	N	7.49	1.54	0.94	5.8	2/3
SW	M	+	-	-	-	23	N	N	N	N	N	5.69	0.94	0.7	4.5	3/3
MH	F	+	-	-	-	30	N	N	N	N	N	9.54	0.56	2.5	6.8	3/3
CO	F	+	-	-	-	28	N	N	N	N	N	9.47	1.04	1.6	7.4	3/3
						28±4						[b] 8.0 [g] ±1.8	1.02 ±0.4	1.4 ±0.8	6.1 [i] ±1.3[n]	
FDB + FH1																
CW[r]	F	+	+	-	-	48	N	Y	Y	N	N	15.3	0.63	1.4	13.6	3/3
RB	F	+	+	-	-	26	N	N	Y	N	N	15.59	0.93	1.35	13.8	3/3
MZ	F	+	+	-	-	21	N	N	N	N	N	11.86	2.16	0.8	10	3/3
MB	F	+	+	-	-	43	N	N	Y	N	N	16.03	1.4	1.0	14.68	3/3
						35±13						[c] 14.7 [gfe] ±1.9	1.4 ±0.7	1,0 ±0.4	13.0 [jk] ±2.0 [lmn]	
FDB + FH1 + FH2																
JJK[s]	M	+	+	+	-	12	Y	N	Y	knees elbows hands in 1979 age 12 almost completely regressed in 1992	AESM (2/6) in 1992	21.66	1.74	0.52	20.79	3/3

Legend:

abcdefghijklmn = Values in the designated groups compared by unpaired two tailed t-tests; $p<0.005$ in all cases except for (i) where $p = 0.0053$. All values expressed as mean ±S.D.

p TC (total cholesterol), TG (triglycerides), HDL (HDL-cholesterol) and LDL (LDL-cholesterol) concentrations expressed as mmol/l

q Apo E genotypes determined as described by Hixson and Vernier (1990)

r CW was the only patient receiving treatment at the time of sampling. Her sole treatment was plasma apharesis. This sample was taken >4 weeks after a plasma apharesis.

s JJK underwent a partial ileal bypass in 1981. His lipid biochemistry values are pre-bypass values.

AESM = Aortic ejection systolic murmur.

Two clearly hyperlipidemic subjects, NM and MK, were negative for these mutations. We then screened NM and her children for the FH Afrikaner-2 and FH Afrikaner-3 mutations. NM and MK were found to be heterozygotes for the FH Afrikaner-2 mutation and JJK was a triple heterozygote for both the FH Afrikaner-1 and FH Afrikaner-2 mutations and FDB. NM's deceased husband must therefore have been an obligate compound heterozygote for FH Afrikaner-1 and FDB. (I subsequently screened all the W family members that had been recruited for the FH Afrikaner -2 and -3 mutations but did not discover any additional cases.)

The availability of such large numbers of individuals with various combinations of LDL receptor mutations and FDB allowed us to examine and compare the clinical and biochemical features of these genetic disorders (Table 6.3). Total cholesterol levels were similar in the FH Afrikaner-1 (8.7±1.5 mmol/l) and FDB heterozygote groups (8.0±1.8 mmol/l) and were clearly higher than normal (5.7±1.1 mmol/l). The LDL-cholesterol levels in the FH Afrikaner-1 (6.9±1.2 mmol/l), FDB (6.1±1.3 mmol/l) and normal groups(3.9±1.2 mmol/l) followed the same trend as the total cholesterol levels. One FDB heterozygote SW had a relatively normal lipid profile with total- and LDL-cholesterol levels of 5.69 mmol/l and 4.5 mmol/l, respectively, at age 23. The total- and LDL-cholesterol levels of the FH-Afrikaner-1/FDB compound heterozygotes of 14.7±1.9 mmol/l and 13.0±2.0 mmol/l, respectively, were higher than those of individuals who had

Table 6.4: Response of CW (a compound heterozygote with FDB and the FH Afrikaner-1 LDL-receptor mutation) to Simvastatin therapy

Date	[c]Dosage	[b]TC	TG	LDL-C	HDL-C
16.03.92 (pre-treatment)		15.3	0.63	13.6	1.4
	20 mg/day				
15.06.92		11.34	0.26	9.6	1.58
		[a](-26%)	(-58%)	(-29%)	(+13%)
	40 mg/day				
27.07.92		9.36	0.58		
		[a](-39%)	(-8%)		

[a] Percentage change in lipid level compared to starting value.

[b] All lipid levels are in mmol/l

[c] Drug was Simvastatin (Zocor, Logos Pharmaceuticals)

just one of the mutations. None of the heterozygotes for either FH Afrikaner-1, FH Afrikaner-2 or FDB had xanthomata or Achilles tendon thickening (Table 6.3) but 3 of the 4 FH Afrikaner-1/FDB compounds had these stigmata. This might be significant as these three compounds were of similar ages to some of the patients with just one mutation. The youngest person in this category, MZ, 21 y, who had the lowest lipid levels, did not exhibit these stigmata.

One of the FH Afrikaner-1/FDB subjects, the proband CW, had been undergoing plasma aphoresis that was stopped before January 1992. On 16.3.92 she was put on Simvastatin (20 mg/day) that was increased 40 mg/day on 15.6.92. Her response to this drug (Table 6.4) was good as far as the percentage drop in total- and LDL-cholesterol was concerned (39%), when compared to that observed in FH and other patients (reviewed by Todd and Goa 1990). However, the final total cholesterol level after 4.5 months of treatment was 9.6 mmol/l, which is the type of total cholesterol concentration that would be expected in an untreated FH heterozygote (Thompson 1989). FH patients on Simvastatin treatment often reduce their total cholesterol levels to 65% or 70% of this value (Todd and Goa 1990).

The clinical features of JJK, the "complex heterozygote" with FH-Afrikaner 1, FH Afrikaner-2 and FDB seem to be similar to those of an FH homozygote. Prior to his partial ileal bypass in 1981 (age 14) he had LDL-cholesterol levels of more than

20 mmol/l (age 12), a corneal arcus and extensive planar and tendon xanthomata. His total cholesterol levels have dropped to 13.7 mmol/l (age 24) and his xanthomata have largely regressed since the partial ileal bypass. He was not on any lipid-lowering medication for at least a year prior to this examination. However, although he is asymptomatic at present, he has a 2/6 aortic ejection systolic murmur and carotid bruits that are evidence of premature atherosclerosis (Table 6.3).

6.3 DISCUSSION

In a North American or European population the frequencies of LDL-receptor mutations and FDB are both about 1/500 (Goldstein and Brown 1989, Innerarity et al. 1990). Thus one would expect to find equal numbers of the two types of defects in hypercholesterolemic patients with xanthomata or thickened tendons from these populations, as both conditions present with similar clinical features (Rauh et al. 1992, Tybjaerg-Hansen et al. 1990).

Three founder LDL receptor mutations account for 74-98% of clinical monogenic hypercholesterolemia (MH) in the Afrikaners (Graadt van Roggen et al. 1991). Although the overall frequency of FH in Afrikaners is about 1/100 (Seftel et al. 1980), the frequency of non-founder LDL receptor mutations is approximately 1/500 ([100 - 74 to 98%] x 1/100), the same as the frequency of FH in most other populations (Goldstein and Brown 1989). One would have expected to find

equal numbers of FDB and LDL receptor mutations in the patients with non-founder mutations with definite MH (hypercholesterolemia with xanthomata or thickened Achilles tendons) if the frequency of FDB in these populations was 1/500. FDB was not detected in 7 Afrikaners with definite MH in our study and a further 13 such patients tested previously (Graadt van Roggen et al. 1991) or in 33 Afrikaners with possible MH or in any of the other categories of hyperlipidemic Afrikaners screened. Thus we feel that FDB is at best only a rare cause of monogenic hypercholesterolemia in this population.

No cases of FDB were detected in the so-called Coloured or Indian populations. The same arguments that were used in the Afrikaners apply here and suggest that FDB is only a rare cause of hypercholesterolemia in these groups.

The FDB mutation probably occurred once on a Caucasian ancestral chromosome and is not the result of multiple mutational events at a CpG dinucleotide (Ludwig and McCarthy 1990). The frequency of this mutation probably varies in different populations as the allele's frequency was likely to have been amplified or reduced by founder effects and random genetic drift. The frequency of FDB in North American and European-Caucasian populations has been estimated to be 1/500 (Innerarity et al. 1990). Although the frequency of this mutation has not been investigated extensively in non-Caucasian populations (Ludwig and McCarthy 1990), FDB has not

been detected in Finns (Hämäläinen et al. 1990) or in Japanese people (Hosking et al. 1991).

There are a number of reasons why FDB might not have been frequently detected in the populations we screened. Firstly, the mutant allele might have been diluted by random drift. Alternatively, in the case of the Indians, it might not be present at all if it is an allele confined to European and North American Caucasians. In the Coloured population the mutant allele might not have been present in the gene pools of their non-Caucasian ancestors and any FDB that might have been introduced from their European ancestors would have been dramatically diluted.

One might argue that FDB is a milder disease than the FH caused by LDL-receptor mutations. Although this was the impression initially (Innerarity et al. 1990), subsequent studies incorporating large numbers of FDB subjects suggest that the two diseases are generally clinically indistinguishable in presentation (Rauh et al. 1992; Tybjaerg-Hansen et al. 1990) and clinical response to lipid lowering therapy (Maher et al. 1991). In any case, FDB was not detected in any of the patients with possible MH or in any of the other hypercholesterolemic groups that were screened in this study.

Two families each of mixed British and Afrikaner origin had FDB. (Because of the uncertain origin of this mutant gene

they were not considered to be Afrikaner). The FDB patients in both families had lipid levels compatible with FH. One patient, SW, in the W family had relatively normal levels, however. Perhaps this is support for the argument that there might be some patients with this mutation that have a milder disease (Innerarity et al. 1990). The variability in the expression of FDB in this subject cannot be explained by the apo E phenotype (see Table 6.3). Perhaps other genetic and environmental influences play a greater role in determining the lipid levels in FDB than in LDL-receptor deficiency, since the former mutation presumably only interferes with LDL clearance, while the latter set of defects affects IDL clearance, LDL production and LDL clearance. Consequently, since FDB presumably affects only the final step of this pathway, it is likely that the variability in the efficiency of the upstream steps is of greater consequence in FDB. Alternatively, the variability of FDB might be modified by polymorphisms in the "normal" apo B allele that affect the clearance rate of the normal LDL. The XbaI polymorphism in the apo B gene has been correlated with differing LDL clearance rates in vivo. The presence of cutting at this site in an individual is associated with increased LDL levels due to decreased catabolism of LDL. Subjects homozygous for XbaI cutting clear autologous LDL 22% less efficiently than their counterparts who are homozygous for apo B alleles that don't have the XbaI site (Houlston et al.1988, Demant et al. 1988).

The most interesting feature of the W family was the presence of 4 patients (+ 1 deceased) that were compounds for FH Afrikaner-1 and FDB. Only 1 case of a compound of an LDL-receptor mutation and FDB has been to my knowledge previously reported (Rauh et al.1991). This group diagnosed LDL-receptor deficiency by determining HMG-CoA reductase activity in skin fibroblasts, thus the genetic nature of the LDL-receptor mutation was not revealed. The conclusion of that group was that the compound was clinically very similar to patients with either defect. The W family allowed the comparison of 4 such compounds with a similar number of FH Afrikaner-1 and FDB heterozygotes.

The clinical features of the FH Afrikaner-1 and FDB heterozygotes were very similar and were clearly less severe than those of the compounds of FH Afrikaner-1 and FDB, both in terms of lipid levels and clinical features. The typical compound seems to have lipid levels that fall between the average levels found in FH heterozygotes (8.9 - 10.8 mmol/l) and those in homozygotes (18.8 to 20.3 mmol/l) (Thompson et al.1989). The range of total cholesterol concentrations quoted by Goldstein and Brown (1989) are 6.5 - 12.9 mmol/l and 15.5 - 25.8 mmol/l for heterozygous and homozygous FH respectively. The total cholesterol values of our compounds were 11.86, 15.3, 15.6 and 16.0 mmol/l. Intuitively one would expect this to be the case: These compounds should be worse off than the FDB heterozygotes since there are not the normal number of receptors present to clear the normal apo-

B100-LDL particles. Similarly they should be worse off than FH heterozygotes as only about 30% of the LDL particles at steady state are competent ligands (Innerarity et al. 1990) for the already reduced receptor complement. However, one would not expect these compounds to be as severely affected as FH homozygotes as a significant proportion of the LDL particles and LDL receptors are normal.

Further evidence for the observation that these FH Afrikaner-1/FDB compounds have a disease that is intermediate in severity between heterozygote and homozygote FH was revealed by one of these patient's responses to Simvastatin therapy. Although her response was good and her total cholesterol levels dropped by 40%, her post-treatment levels were equivalent to those of an untreated FH or FDB heterozygote and certainly higher than one would expect from a treated FH or FDB subject (Todd and Goa 1990, Maher et al. 1991) (Table 6.4).

The clinical presentation of the "complex" heterozygote that had both FH Afrikaner-1 and -2 mutations and FDB was similar to that of a normal homozygote with LDL-receptor mutations (Thompson et al. 1989). It is not possible at present to say whether this subject is worse off with the apo B mutation than he might have been without it. The FH Afrikaner-1 mutation does express a significant number of functional receptors on human skin fibroblasts but the FH Afrikaner-2 mutation is practically a null mutation due to its very short

half-life (Fourie et al. 1988). Thus, a heteroallelic heterozygote for these two mutations would have some residual LDL-receptor activity (Fourie et al. 1988). The greater the residual LDL receptor activity is, the greater will be the liability of the additional presence of the FDB mutation. In theory, this could be assessed by comparing the ratio of mutant apo B to normal apo B in LDL particles from the FDB, FDB/FH Afrikaner-1 and FH Afrikaner-1/FH Afrikaner-2/FDB subjects. (This type of experiment could be enhanced by treatment of this subject with Simvastatin that would theoretically upregulate his residual functional receptors.) If the ratio of mutant to normal apo B were still significantly greater than 1:1 in the patient with 2 LDL-receptor mutations and FDB, then one would assume that there was residual LDL-receptor activity and that he was worse off having the additional apo B defect. The clinical sequelae would probably not be more severe than those in a patient with 2 null LDL-receptor alleles, as it is unlikely that any significant normal apo B-100-mediated LDL clearance occurs in the absence of LDL receptors.

The presence of the combination of both apo B and LDL-receptor gene defects in individuals in this family underlines the importance of testing for both classes of defects in the appropriate populations and patient groups at risk. Populations that have a particularly high frequency of either FDB or LDL-receptor mutations are more likely to contain individuals with both types of defect. This scenario

should be considered in "FH heterozygotes" with slightly higher than expected lipid levels that do not drop as close to normal as one would expect on Simvastatin or similar therapy. The identification of one of these 2 causes of monogenic hypercholesterolemia should not be considered completely diagnostic unless the other class of defect has been ruled out.

CHAPTER 7

CONCLUSION

One of the hallmarks of progress in medical science has been in the way the concept of disease has been understood. Initially, most diseases were merely symptom complexes that behaved distinctly. Then followed a mechanistic elucidation of the pathological processes that caused the symptom complexes. In certain cases, it was found that different and distinct pathological processes resulted in final symptom complexes that were similar. The fact that two or more distinct "diseases" can cause similar symptom complexes was an important step forwards. Often the different "diseases" had different cures and/or prognoses. The converse situation also arose where two seemingly unrelated symptom complexes had the same pathological cause and could be treated together. Thus, it was important to understand diseases as pathological processes and not merely as symptom complexes.

The recent explosion in the knowledge of the molecular basis of disease has introduced a further level of understanding of many disease processes: the precise mutations and the genes involved in diseases can be identified.

The understanding of familial hypercholesterolemia has followed this pathway. In 1873 Fagge (1873) described FH associated with xanthomas in a multigenerational family and hypothesised that the fatty deposits in the skin were related

to those in the arteries. In 1939 Carl Müller (1939) consolidated the understanding of this symptom complex in his description of Familial Hypercholesterolemia as a fairly common dominant inborn error of metabolism that resulted in xanthomata, raised plasma cholesterol concentrations and an increased frequency of early heart attacks. The work of Khachadurian in the 1960s (Khachadurian 1964) elucidated further aspects of this disease by clearly demonstrating differences in clinical severity between homozygotes and heterozygotes. The observations of Gofman and colleagues (1934) that an elevation of LDL particles caused the hypercholesterolemia in FH and the demonstrations by Fredrickson et al. (1967) that this was due to impaired LDL catabolism, marked the stage when the pathological processes underlying FH began to be understood.

The work of Brown and Goldstein that showed that FH was caused by mutations in the gene coding for the cell-surface receptor for LDL, led to the understanding of this pathological process at the genetic level (Brown et al. 1974, Brown and Goldstein 1974). Today we know that many different mutations in the LDL-receptor gene (Hobbs et al. 1990) and the mutation at codon 3500 in apo B100 (Rauh et al. 1992) cause this clinical picture. One could argue that the importance today of knowing the precise nature of the mutations involved in individual patients is probably only of academic interest . However, considerable heterogeneity of FH exists and is perhaps most clearly demonstrated by the

susceptibility to heart disease of patients homozygous for null mutations relative to those with receptor-defective LDL receptor mutations (Yamamoto et al. 1989, Goldstein and Brown 1983). There also seems to be considerable heterogeneity in the expression of FDB that is as yet unexplained (Innerarity et al. 1990). Although at the moment there does not seem to be much difference in the therapeutic response of FDB and LDL-receptor mutations to cholesterol-lowering therapy (Maher et al. 1991), such a difference might exist with newer drugs that are developed since these "diseases" cause hypercholesterolemia through subtly distinct mechanisms.

Perhaps the next step in the understanding of disease is going to be progress in the elucidation of gene-gene and gene-environment interactions that result in variation in disease expression and cause polygenic conditions.

The main aim of this thesis was to try to identify and understand the mutations responsible for familial hypercholesterolemia in the South African Indian population. At least four different LDL receptor mutations have been identified: The Pro_{664}-Leu, Asp_{69}-Tyr, Glu_{119}-Lys point mutations and the "Null" mutation in NS.

The Pro_{664}-Leu mutation, FH Zambia, was initially described and characterised by Soutar and Knight as a mutation that resulted in the LDL receptor having an impaired affinity for both LDL and ß-VLDL (Knight et al. 1989, Soutar et al. 1989).

This finding seemed very interesting as the point mutation was in Repeat C in the EGF-precursor homology domain, more than 300 amino acids away from the ligand-binding domain of the receptor. The availability of fibroblasts from two patients who were homozygous for this mutation allowed us to re-examine this mutant protein's behaviour. Our studies suggested that this mutation does not affect the mutant receptor's ability to bind either ß-VLDL or LDL. The mutation resulted in the rapid degradation of mature receptors that could account for the decreased cell-surface population of normally functional receptors. This presumably caused FH in these subjects.

The patient D was a heteroallelic homozygote with the Asp_{69}-Tyr (ligand-binding repeat 2) and Glu_{119}-Lys (repeat 3) point mutations (both at highly conserved sites). The impaired ability of mutant receptors on his fibroblasts to bind LDL was consistent with the results of experiments done using artificial constructs with nearby mutations or deletions of the same ligand-binding repeats (Russell et al. 1990). Natural mutations at homologous sites in different ligand-binding repeats suggest that both asp_{69} and glu_{119} play important functional or structural roles within their repeats (Leitersdorf et al. 1990; Leitersdorf E, Graadt van Roggen F, van der Westhuyzen D and Coetzee G, personal communication.) Thus, these amino acid substitutions are believed to be the mutations causing FH in this patient.

The patient NS did not express any detectable LDL-receptor protein. The mRNA levels from his fibroblasts were low when compared to normal but the message size was approximately normal. The absence of any abnormality in the promoter of his gene suggests that this mutation is probably a point mutation or a small insertion or deletion that creates a stop codon.

JL, a Black patient belonging to the Pedi tribe, was also investigated as an FH homozygote whose fibroblasts expressed very low numbers of LDL-receptors. The underlying cause for the receptor deficiency in this patient was shown to be at the synthetic level. This was confirmed by the finding of low LDL-receptor mRNA levels. DNA sequence analysis of JL's LDL-receptor promoter revealed that he was a heteroallelic homozygote and that one of his alleles had a 3 b.p. deletion in the Sp1 repeat 1 (G.Zuilani, H.Hobbs and L.F. de Waal, personal communication).

Indian patients referred to us by Lipid clinics from all over the country were screened for the three point mutations that were identified in this group and for FDB (Fig. 7.1). It was not surprising that we detected no major founder LDL-receptor mutation in the Indians, as they are descended from a large initial immigrant population (150 000) of diverse geographical, cultural and religious origins and have only been in South Africa for 3-4 generations. However, it was interesting that we found four unrelated families with the

Table 7.1: Summary of Results of Indian Patients Screened for LDL-Receptor Mutations and FDB (Familial Defective Apolipoprotein B-100)

MUTATION	NO. OF PATIENTS WITH MUTATION	PATIENTS NEGATIVE FOR MUTATION				
		DEFINITE FH[a]	POSSIBLE FH[b]	TYPE IIB[c]	MIXED HYPER-LIPIDEMIA[d]	MILD HYPER-CHOLESTEROLEMIA[e]
Pro_{664} to Leu (FH-Zambia)	4	7	13	8	5	4
Asp_{69}-Tyr (D-exon 3)	1[f]	8	10	7	5	6
Glu_{119}-Lys (D-exon 4)	1[f]	8	10	7	5	6
NS (Null)[g]	1	-	-	-	-	-
FDB	0	7	13	8	5	3

Legend:

a Definite FH: Total Cholesterol >7.5 mmol/l or LDL-cholesterol >5.2 mmol/l with xanthomata or thickened achilles tendons in patient or 1st degree relative.
b Possible FH: Total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5.2 mmol/l with triglycerides < 2.3 mmol/l
c Type IIb: Total cholesterol > 7.5 mmol/l or LDL-cholesterol > 5.2 mmol/l with triglycerides > 2.3 mmol/l but < 4.5 mmol/l.
d Mixed hyperlipidemia: Total cholesterol > 7.5 mmol/l and triglycerides > 4.5 mmol/l
e Mild hyperlipidemia: Total cholesterol > 6 mmol/l
f Same patient has both mutations (a heteroallelic homozygote).
g Gene defect not known.

Pro_{664}-Leu mutation. All four families were Muslim and traced their origins to the vicinity of Surat in the Gujerat province of India. The patient MM that was described by Soutar and Knight (Soutar et al. 1989, Knight et al. 1989) and the subsequent Indian identified with this mutation in London (King-Underwood et al. 1991) were also of Gujerati Muslim origin. It is thus likely that there is a founder pocket of this mutation in that area of India.

The SSCP technique was used initially to detect and subsequently to screen for the Asp_{69}-Tyr and Glu_{119}-Lys mutations among Indian lipid clinic patients. Except for the index case, no other patients with these mutations were identified. There are still a few Indian families with definite FH (raised cholesterol levels and xanthomata) whose defects are not known. It is possible that some of them have the as yet unidentified "Null" mutation found in NS but the more likely scenario is that there are many different LDL-receptor mutations in this group reflecting their diverse geographical origins.

To the best of my knowledge, there have been no previous studies that have investigated FDB in South Africa. No FDB was detected in any of the Indian patients that were screened. The absence of this mutation in these patients suggests that the majority of clinical FH in South African Indians can probably be accounted for by mutations in the LDL-receptor gene and that the frequency of FDB is probably

lower than that found in European and North American Caucasians.

We also screened a number of Afrikaner, so-called Coloured and Jewish patients for the FDB mutation. Patients who were positive for founder LDL-receptor mutations were considered separately from those who were negative. The absence of FDB in the FH patients who didn't have the LDL-receptor founder mutations was evidence that this mutation is not a major contributor to clinical FH in the Afrikaners and the so-called Coloureds. The frequency of both FH and FDB in European populations have both been estimated at 1/500 (Goldstein and Brown 1989, Innerarity et al. 1990). We thus would have expected similar numbers of LDL-receptor mutations and FDB to cause "FH" in our subjects that did not have founder mutations. It is thus possible that FDB might be under-represented in the Afrikaners and so-called Coloureds compared to the European populations. (The number of definite Jewish FH patients that were screened for FDB that were negative for the FH Piscataway mutation was too small to allow us to comment).

As part of this screening program we identified two families of mixed English and Afrikaner descent that had the FDB mutation. One of the families was particularly interesting, as in addition to subjects who were heterozygous for FDB, there were heterozygotes for the FH Afrikaner-1 and FH Afrikaner-2 LDL receptor mutations, a number of compound

heterozygotes for FDB and FH Afrikaner-1 mutations and one subject who was a heteroallelic homozygote for the FH Afrikaner-1 and FH Afrikaner-2 mutations and heterozygous for FDB. The availability of such a diversity of mutations in one family allowed us to compare the clinical severity of each alone and in combination. The subjects who were heterozygous for LDL-receptor mutations were clinically similar to those with FDB. The subjects who had a combination of an LDL-receptor defect and FDB were clearly worse off than those with either defect, in contrast to the previous report of such a patient (Rauh et al. 1991). However these subjects were not as severely affected as typical FH homozygotes. The co-inheritance of both ligand and receptor defects might be more common than one might imagine especially in populations that have an enrichment of one or both of these causes of monogenic hypercholesterolemia.

In conclusion, 4 different LDL-receptor mutations were identified in South Africans of Indian origin. There was no evidence of a founder effect amplifying the frequency of any of these mutations in South Africa. However, the FH Zambia mutation is likely to be common in Muslims of Gujerati origin. FDB was not detected in any Indian, Afrikaner or Coloured patients with Monogenic Hypercholesterolemia and is probably not as prevalent in these populations as it is in European and North American Caucasians.

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